Full Paper

Synthesis of Some Novel D-Glucuronic Acid Acetylated Derivatives as Potential Anti-Tumor Agents

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A structurally diverse series of $\Delta^{4,5}$-uronamide derivatives have been chemically synthesized starting from D-glucuronic acid itself by means of acetylation, activation, amide bond formation and base-catalyzed elimination protocols. Structure elucidation for all products along with optimization of the synthetic steps is described. The synthesized compounds were evaluated for their *in-vitro* anti-tumor activity against MCF-7, TK-10 and UACC-62 cell lines. The compounds **5**, **11**, **13**, **15** and **16** were the most active against TK-10 cell line. On the other hand, the most active compounds against the MCF-7 cell line were **11** and **15**. However, compounds **5**, **7**, **11**, **13**, **15** and **16** were the most active against the UACC-62 cell line.

Keywords: Amide linkage / Anti-tumor / D-Glucuronamide / D-Glucuronic acid / MCF-7 / TK-10 / UACC-62

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Introduction

Cancer has been a major lethal threat worldwide till date. This malady is not a single ailment, but rather a collection of hundreds of different diseases as a result of uncontrolled cell proliferation. Chemotherapy is a major cancer treatment besides surgery and radiation therapy. The majority of the chemotherapeutic drugs that are currently in use are organic drugs or natural products. Some examples of natural products as potential anti-tumor agents are illustrated in (Fig. 1). The synthesis of new compounds and testing their biological and pharmacological activities are the major goals of drug development projects [1]. Sialic acids are involved in a range of biological processes including cell-cell, cell-microbe interactions [2–4], masking effects for cell surface antigen [5, 6], differentiation of cells [7–9], and neoplastic transformation [10–12]. These biological importances were further confirmed by the discovery of a wide range of proteins that recognize them. The importance of sialic acids in these processes, especially with respect to human disease states, has led to interest in the synthesis of natural and modified sialic acids [13, 14] both as probes of sialic acid-recognizing proteins, and as potential glycopharmaceuticals. The 2,3-dehydro-2-deoxy-sialic acids Neu5Ac2en, Neu5Gc2en, and their acetates are widely distributed in nature [15] and display interesting biological activities as transition state inhibitors of the enzyme, sialidase [16]. A 2,3-dehydro-sialic acid, such as Neu2en5Ac, Neu2en5Gc and Kdn2en mimetics, is essentially derived from 4,5-unsaturated-D-glucopyranosiduronic acid (Fig. 2). Combination of non-carbohydrate moieties with sialic acid derivative would be anticipated to give rise to selective interaction towards target biological molecules and target tumor cells [17–20].

Research within our group has been recently directed towards the preparation of a series of indole-pyrimidine (Meridianin D analogues) and phthalazinone-amino acid conjugates. These conjugates were tested as anti-tumor agents and showed moderate activities against caucasian breast adenocarcinoma (MCF-7) cell line [21–24]. As a part of our ongoing research on drug discovery, we have been interested

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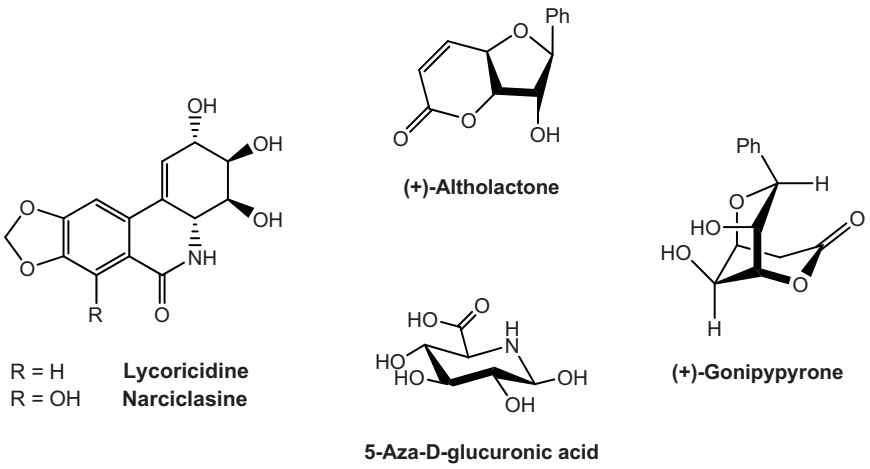


Figure 1. Examples of natural products containing sugar ring as potential antitumor agents.

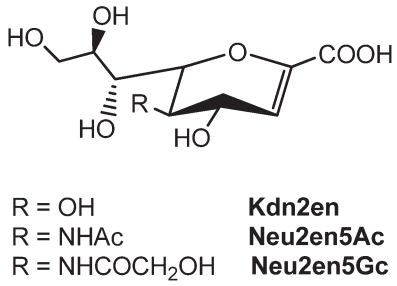


Figure 2. Structures of Neu5Ac2en, Neu5Gc2en and Kdn2en.

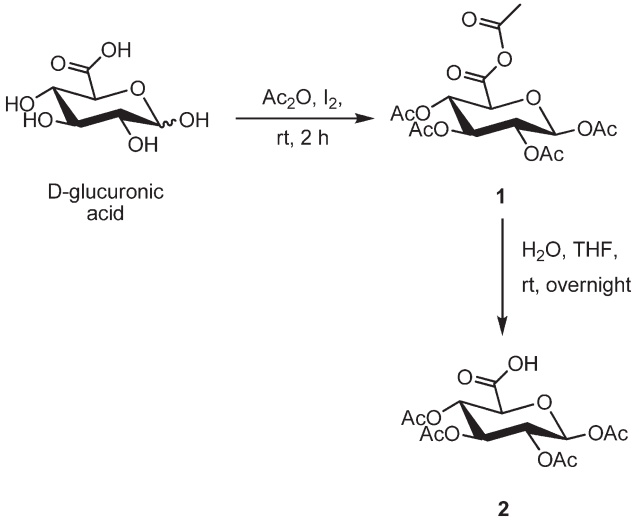
in developing a family of compounds containing an acetylated sialic acid nucleus through a facile and rapid synthetic pathway to new drug-like small organic molecules.

Results and discussion

Synthesis of the saccharide conjugates

Studies were initiated by the acetylation of the commercially available D-glucuronic acid using acetic anhydride and a catalytic amount of iodine as an acetyl transfer reagent [25] to give the 1,2,3,4-tetra-*O*-acetyl mixed anhydride **1** in 98% yield (Scheme 1). The formation of **1** was confirmed by ^{1}H-NMR spectroscopy. In particular, the acetoxy methyl signal that appeared at relatively low field (2.28 ppm) indicates the existence of the $COOCOCH_3$ group, in addition to the higher field signals observed at 2.13 (3H), 2.05 (6H) and 2.04 ppm (3H) which corresponds to the four *O*-acetyl groups.

Compound **1** was subjected to reaction with water to afford the corresponding 1,2,3,4-tetra-*O*-acetyl-β-D-glucuronic acid **2** in 99% yield (Scheme 1). The formation of **2** was proven using ^{1}H-NMR which revealed the absence of the acetoxy methyl signal at 2.28 ppm and the appearance of a broad signal at 3.44 ppm corresponding to the carboxyl group proton [25].



Scheme 1. Acetylation of D-glucuronic acid.

Glucuronates derivatives were reported to readily undergo β-*cis*-elimination to give the corresponding 4,5-unsaturated species by treatment with a base [26–32]. The electron-withdrawing effect of the carbonyl group makes the α-proton (H-5) sufficiently acidic to be removed by a base. It has been proposed that there are three steps involved in the elimination. Firstly, removal of H-5 forming a carbanion, followed by conformational inversion, and finally, elimination to give the 4,5-unsaturated derivative [26] (Fig. 3).

On this basis, a straightforward one pot procedure was employed starting from acid **2** in which, in addition to the amide bond formation, a base-promoted elimination of acetic acid was achieved. The coupling of **2** with different amines in the presence of ethyl chloroformate and TEA at -20°C produced, after extractive work-up, the desired 4,5-unsaturated amides **3**, **5**, **7**, **9**, **11** and **13** along with the non-elimination

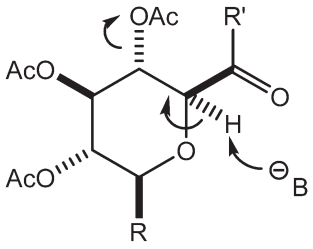
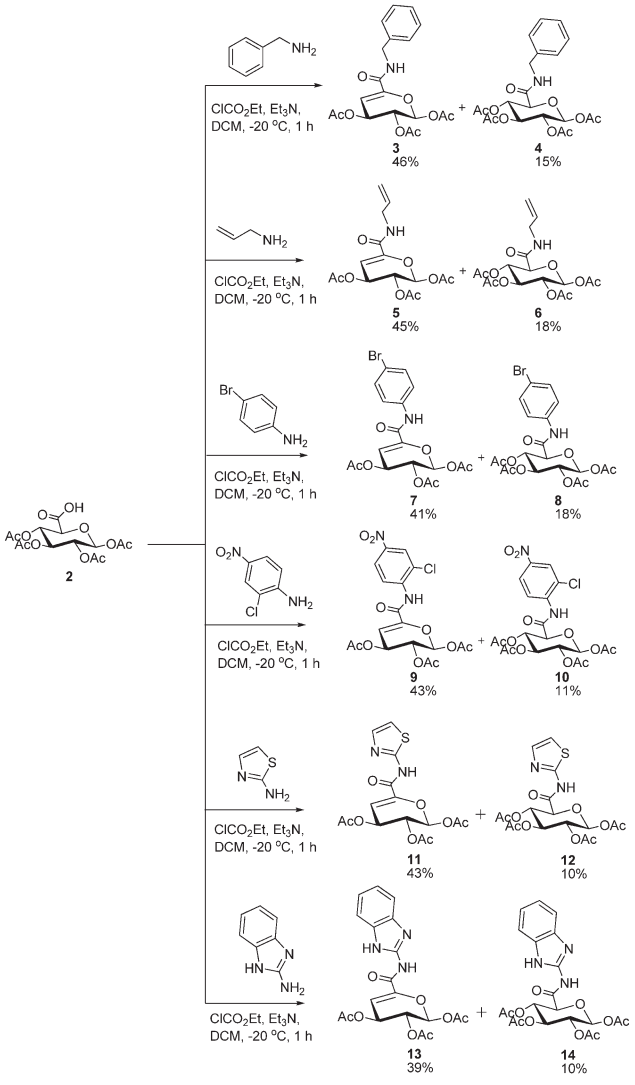


Figure 3. β-Elimination in D-glucuronic acid derivatives.



Scheme 2. Reaction of **2** with benzyl, allyl, substituted aromatic and heterocyclic aromatic amines.

products **4**, **6**, **8**, **10**, **12** and **14** which were also identified (Scheme 2). Fortunately, these products could be separated by a slow silica gel column chromatography.

By using benzyl or allyl amine, the corresponding *N*-(benzyl)- (**3**) or *N*-(allyl)-1,2,3-tri-*O*-acetyl-$\Delta^{4,5}$-β-D-glucopyranuronamide (**5**) were obtained, respectively. The structural assignment for **3** and **5** were confirmed on the basis of their NMR spectral data. The ^{1}H-NMR spectrum of compound **3** showed the anomeric proton at 6.21 ppm. The other H-3 and H-2 ring protons appeared at 5.17 and 5.04 ppm, respectively, while the olefinic proton appeared at 6.18 ppm. The remaining three acetoxy groups appeared as three singlets at 1.99, 1.98 and 1.97 ppm, while the methylene protons resonated at 4.39 ppm.

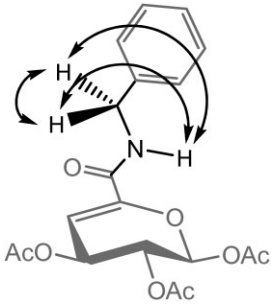


Figure 4. Spin-spin coupling interactions of the methylene protons.

The splitting pattern of the methylene group was ddd (double of doublets for each methylene proton) which corresponds to an ABX system and it was reduced to a double of doublets after proton exchange experiment. This was accounted by the presence of asymmetric centers in the vicinity of the methylene group which makes a 'non-equivalence' between the two methylene protons and in turn a diastereotopic effect will be reflected in the ^{1}H-NMR spectrum and the CH_2 group will appear as a double of doublets (doublet for each methylene proton). Further coupling with the neighboring amide NH proton will give the observed ddd pattern (Fig. 4).

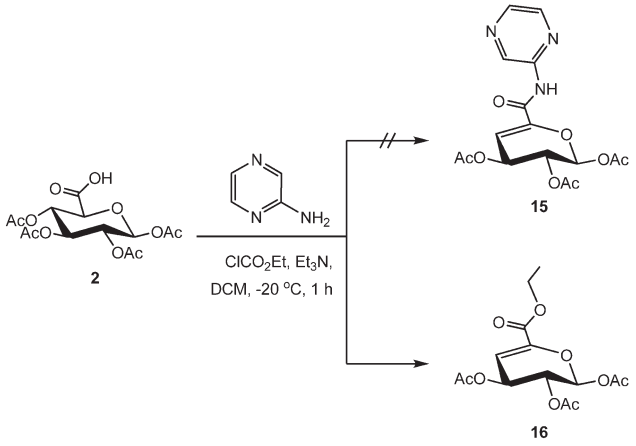
Additionally, ^{13}C-NMR interpretation assisted with DEPT confirmed the structure of **3** by the observation of a signal at 88.7 ppm corresponding to C-1 atom. The other two signals at 67.5 and 64.1 ppm corresponds to C-2 and C-3, respectively, while the two signals at 144.8 and 103.8 ppm corresponds to C-5 and C-4 olefinic carbons, respectively. The four signals appearing in the 170.0–160.6 ppm region are due to the four carbonyl carbon atoms, while the signals at 21.2 and 21.1 are due to the acetate methyl carbons. The benzyl group was also observed in the ^{13}C-NMR by the appearance of four signals at 137.9, 129.2, 128.4 and 128.1 ppm corresponding to the phenyl ring carbons, and a signal at 43.9 ppm corresponding to the methylene carbon atom. On the other hand, the ^{1}H NMR spectrum for compound **5** showed the anomeric proton at 6.30 ppm with as spin-spin coupling constant of 3.1 Hz which is typical axial–quasi-axial coupling between H-1 and H-2 protons [33], while the other H-2 and H-3 ring protons appeared at 5.14 and 5.26 ppm, respectively. Additionally, ^{13}C-NMR interpretation assisted with DEPT confirmed the structure of **5** by the observation of a signal

at 88.2 ppm corresponding to C-1 atom. The other two signals at 67.0 and 63.5 ppm corresponds to C-2 and C-3, respectively, while the two signals at 144.2 and 103.1 ppm corresponds to C-5 and C-4 olefinic carbons, respectively. The four signals appearing in the 169.6–160.0 ppm region are due to the four carbonyl carbon atoms, while the signals at 20.7 and 20.6 are due to the acetate methyl carbons. The allyl carbons were also observed by the appearance of two signals at 133.2 and 116.9 ppm corresponding to the two olefinic carbons and a signal at 41.7 ppm for the methylene carbon.

By using 4-bromoaniline or 2-chloro-4-nitroaniline as aromatic amines, the corresponding *N*-(4-bromophenyl)- (**7**) or *N*-(2-chloro-4-nitrophenyl)-1,2,3-tri-*O*-acetyl-$\Delta^{4,5}$-β-D-glucopyranuron-amide (**9**) were obtained, respectively. Again, the structural assignment for **7** and **9** were confirmed on the basis of their NMR spectral data. In particular, the ^{1}H-NMR for compound **7** showed the anomeric proton at 6.37 ppm overlapped with the olefinic proton H-4, while the other H-2 and H-3 ring protons appeared at 5.16 and 5.28 ppm, respectively. Additionally, ^{13}C-NMR interpretation confirmed the structure of **7** by the observation of a signal at 88.3 ppm corresponding to C-1 atom, while the two signals at 143.9 and 104.3 ppm corresponds to C-5 and C-4 olefinic carbons, respectively. The ^{1}H-NMR for compound **9** showed the anomeric proton at 6.44 ppm with as spin-spin coupling constant of 4.6 Hz which is typical axial-quasi-axial coupling between H-1 and H-2 protons [33]. The olefinic proton appeared at 6.41 ppm, while the other H-2 and H-3 ring protons appeared at 5.20 and 5.32 ppm, respectively.

By using 2-aminothiazole or 2-aminobenzimidazole as heterocyclic aromatic amines, the corresponding *N*-(thiazol-2-yl)- (**11**) or *N*-(1*H*-benzimidazol-2-yl)-1,2,3-tri-*O*-acetyl-$\Delta^{4,5}$-β-D-glucopyranuronamide (**13**) were obtained, respectively, and the structural assignments were confirmed on the basis of their NMR spectral data. In particular, the ^{1}H-NMR for compound **11** showed the anomeric proton overlapped with the olefinic proton in the 6.34–6.32 ppm region. The other H-2 and H-3 ring protons appeared at 5.12 and 5.22 ppm, respectively. Additionally, ^{13}C-NMR interpretation confirmed the structure of **11** by the observation of a signal at 88.8 ppm corresponding to C-1 atom, while the two signals at 143.6 and 106.1 ppm corresponds to C-5 and C-4 olefinic carbons, respectively. The three thiazole ring carbons were also observed at 158.1, 138.4 and 115.0 ppm. On the other hand, the ^{1}H-NMR for compound **13** showed the anomeric proton at 6.35 ppm with as spin-spin coupling constant of 4.6 Hz which is typical axial-quasi-axial coupling between H-1 and H-2 protons [33].

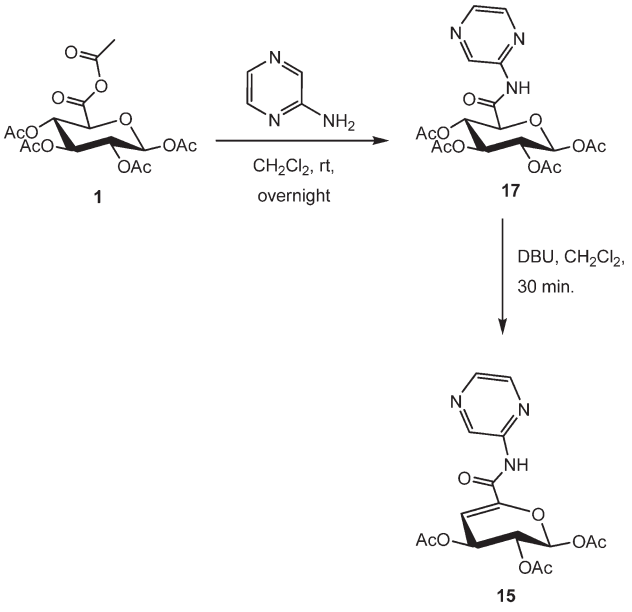
Interestingly, the attempt to use 2-aminopyrazine as the amine in this reaction gave a single reaction product as indicated by TLC, but after purification and spectral data inspection, it was found that the reaction did not give the desired *N*-(pyrazin-2-yl)-1,2,3-tri-*O*-acetyl-$\Delta^{4,5}$-β-D-glucopyranuronamide (**15**), but instead it gave ethyl 1,2,3-tri-*O*-acetyl-$\Delta^{4,5}$-β-D-glucopyranuronate (**16**) (Scheme 3). The formation of **16** was an unexpected result, and the mechanism of its formation requires further investigation.



Scheme 3. Reaction of **2** with 2-aminopyrazine.

The structure of compound **16** was assigned on the basis of its NMR spectral data. In particular the ^{1}H-NMR spectrum revealed nine signals; a quartet at 4.31 ppm and a triplet at 1.34 ppm corresponding to the ethyl group, three signals at 6.41, 5.23 and 5.15 ppm corresponding respectively to H-1, H-3, H-2 ring protons and a signal at 6.27 ppm corresponding to the olefinic ring proton. Additionally, the ^{13}C-NMR spectra confirmed the suggested structure of **16**. The two signals at 62.0 and 14.13 ppm were assigned for the ethyl carbon atoms, while the signal at 161.2 ppm was assigned for the ethyl ester carbonyl carbon.

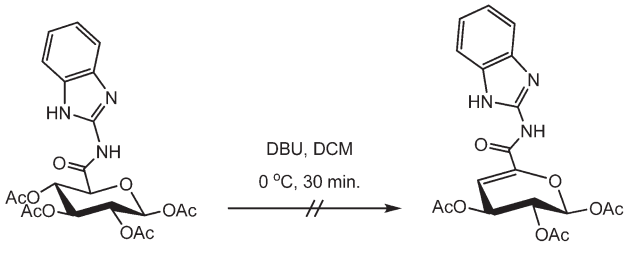
The focus was shifted to find an alternative route for the preparation of *N*-(pyrazin-2-yl)-1,2,3-tri-*O*-acetyl-$\Delta^{4,5}$-β-D-glucopyranuronamide (**15**) and a two step procedure was suggested (Scheme 4). *N*-(Pyrazin-2-yl)-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranuronamide (**17**) was obtained from the reaction of 2-aminopyrazine and **1** in anhydrous DCM at room temperature. After product purification, a solution of **17** in anhydrous DCM maintained at 0°C was then treated with DBU. After 30 min, TLC analysis of the reaction mixture indicated a complete consumption of the starting material and a clean conversion to a product with a higher R_f. To work-up the reaction, the solvent was removed *in vacuo* and the residue was subjected to column chromatography to give the desired product **15**. The NMR spectroscopic analysis of the product confirmed the elimination as the ^{1}H-NMR spectrum showed an olefinic H-4 resonates at ~6.3 ppm and the absence of resonances for H-5. Additionally, the ^{13}C-NMR showed downfield signals for olefinic carbons at 105.4 ppm and 143.6 ppm which are consistent with the C-4–C-5 double bond. Additionally, both the ^{1}H-NMR and ^{13}C-NMR showed signals which corresponds to only three acetyl groups. The DBU



Scheme 4. Preparation of compound **15.**

promoted elimination procedure was also tried for the preparation of the other 4,5-unsaturated compounds (**3**, **5**, **7**, **9**, **11** and **13**) from the corresponding 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranuronamides which successfully produced the same compounds as a single product in moderate yields except for *N*-(1*H*-benzimidazol-2-yl)-1,2,3,4-tetra-*O*-acetyl-β-D-glucuronamide (**14**) in which the reaction suffered from series of complications (Scheme 5).

β-Elimination was accompanied by a conformational change of the ring from $^4C_1$ chair to half-chair to accommodate the newly formed double bond. This was reflected in the ^{1}H-NMR spectrums of all products through the reduction in the magnitude of coupling between ring protons that are no longer in an axial–axial relationship with their neighbors. Further indication for β-elimination was provided by the observed downfield shift of H-4 signal and the disappearance of the H-5 signal. The conformational change was also reflected in ^{13}C-NMR spectrum by the upfield shifts of signals corresponding to C-1, C-2 and C-3. Furthermore, the appearance of two downfield signals is consistent with C-4–C-5 double bond carbons. The ring conformation of each product was determined by comparing their coupling constants with those of known glycosides of hex-4-enopyranuronate [29, 34]. The coupling between H-2 and H-3 was small ($J_{2,3} < 3.0$ Hz), and within the range expected for 4,5-unsaturated glucuronides having $^1H_2$ conformation. Further, long range (W) coupling is also observed between H-2 and H-4, as well as, between H-1 and H-3 in some products, which is also consistent with the $^1H_2$ conformation [29] (Fig. 5).



Scheme 5. Reaction of *N*-(1*H*-benzimidazol-2-yl)-1,2,3,4-tetra-*O*-acetyl-β-D-glucuronamide with DBU.

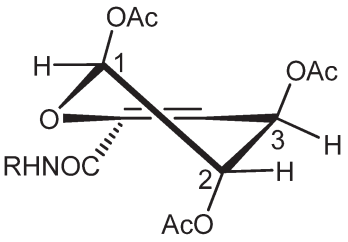


Figure 5. The concluded conformation for the prepared 4,5-unsaturated glucuronamide derivatives.

Anti-tumor evaluation and discussion

The newly synthesized compounds were examined for *in-vitro* activity against several human cancer cell lines, including renal adenocarcinoma (TK-10), human breast adenocarcinoma (MCF-7) and human melanoma (UACC-62). Three response parameters (GI_{50}, TGI, and LC_{50}) were calculated for each cell line. The GI_{50} value (growth inhibitory activity) corresponds to the concentration of the compounds causing 50% decrease in net cell growth, the TGI value (cytostatic activity) is the concentration of the compounds resulting in total growth inhibition and the LC_{50} value (cytotoxic activity) is the concentration of the compounds causing net 50% loss of initial cells at the end of the incubation period. Table 1 lists the GI_{50}, TGI and LC_{50} values obtained for all tested compounds.

Regarding the activities of the 4,5-unsaturated derivatives (**3**, **5**, **7**, **9**, **11**, **13**, **15** and **16**) in Table 1, compounds **7**, **9**, **11**, **15** and **16** showed a growth inhibitory activity on the three mentioned tumoral cell lines at the tested doses. However, compounds **3**, **5** and **13** showed only an inhibitory effect against the growth of the TK-10 and UACC-62 cells, but did not posses any activity on the MCF-7 cell lines. The compounds **5**, **11**, **15** and **16** were the most cytotoxic on TK-10 (GI_{50} = 0.23, 1.51, 2.38 and 0.14 μM, respectively). On the other hand, the most compounds which were affected on the MCF-7 cell line were **11** and **15** (GI_{50} = 0.32 and 3.04 μM, respectively). However, all tested 4,5-unsaturated derivatives, with exception of compounds **3** and **9**, showed a high growth inhibitory activity against the UACC-62 cell line. The growth

Table 1. Compound concentrations (μM) required to cause different inhibitions.

Compound number	Inhibition parameter	Cell Line		
		TK-10	MCF-7	UACC-62
3	GI_{50}	84.96 ± 13.48	>100	24.38 ± 2.01
	TGI	>100	>100	>100
	LC_{50}	>100	>100	>100
5	GI_{50}	0.23 ± 0.01	>100	0.036 ± 0.01
	TGI	>100	>100	0.71 ± 0.03
	LC_{50}	>100	>100	15.06 ± 1.97
7	GI_{50}	14.43 ± 3.31	99.73 ± 21.63	2.14 ± 0.12
	TGI	34.63 ± 8.22	>100	8.55 ± 0.50
	LC_{50}	83.05 ± 6.19	>100	34.12 ± 6.18
9	GI_{50}	32.83 ± 6.81	25.71 ± 2.77	16.09 ± 3.17
	TGI	72.13 ± 12.07	>100	36.31 ± 7.68
	LC_{50}	>100	>100	81.91 ± 16.00
11	GI_{50}	1.51 ± 0.80	0.32 ± 0.31	0.803 ± 0.21
	TGI	3.44 ± 0.10	1.06 ± 0.10	2.57 ± 0.18
	LC_{50}	7.85 ± 1.18	3.55 ± 0.18	8.22 ± 1.92
13	GI_{50}	6.00 ± 0.09	>100	1.53 ± 0.03
	TGI	>100	>100	11.46 ± 2.81
	LC_{50}	>100	>100	85.67 ± 11.50
15	GI_{50}	2.38 ± 0.04	3.04 ± 0.09	0.62 ± 0.02
	TGI	7.71 ± 1.50	80.87 ± 19.11	4.67 ± 0.99
	LC_{50}	25.00 ± 5.16	>100	24.75 ± 4.75
16	GI_{50}	0.14 ± 0.05	9.03 ± 1.11	0.18 ± 0.02
	TGI	>100	>100	>100
	LC_{50}	>100	>100	>100

The range of doses assayed was 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M. Results are mean ± S.E.M. ($n = 3$).

of TK-10 cells was totally inhibited by **7**, **9**, **11** and **15** (TGI = 34.63, 72.13, 3.44, and 7.71 μM, respectively). At the same time, all the tested 4,5-unsaturated derivatives, with exception of compounds **3** and **16**, showed a cytostatic and a cytotoxic activities against the UACC-62 cell line. Compounds **11** and **15** demonstrated a cytostatic activity against the MCF-7 cell line with TGI values of 1.06 and 80.87 μM, respectively. Furthermore, only **11** produced a cytotoxic activity (LC_{50}) against MCF-7 cell line at the dose of 3.55 μM.

Summary

A highly efficient and practical method was described for the preparation of $\Delta^{4,5}$-uronamide derivatives according to standard protocols. The newly synthesized compounds were examined for *in-vitro* activity against several human cancer cell lines, including TK-10, MCF-7 and UACC-62. The compounds **5**, **11**, **13**, **15** and **16** were the most active against TK-10 cell line. On the other hand, the most active compounds against the MCF-7 cell line were **11** and **15**. However, compounds **5**, **7**, **11**, **13**, **15** and **16** were the most active against the UACC-62 cell line.

Experimental

Chemistry

General methods

All starting materials and reagents were purchased from Sigma-Aldrich, BDH and Fluka and used without further purification. All solvents were either of analytical grades or dried and distilled immediately prior to use: DCM from calcium hydride and THF from sodium/benzophenone. All of the reactions were performed using oven-dried glassware. Melting points were measured on a Stuart-SMP10 melting point apparatus and are uncorrected. TLC was performed using Merck precoated Silica gel 60 F254 aluminum sheets (20 × 20 cm, layer thickness 0.2 mm) and spots were visualized by UV (254 nm), $KMNO_4$ solution and/or charring with H_2SO_4/EtOH (5% v/v). Column chromatography was carried out on Silica Gel 60 (particle size 0.063–0.200 mm, 70–230 mesh ASTM, Merck) using the specified eluents. All reaction products were stored refrigerated under 4°C. NMR spectra were recorded with JEOL ECA-500, JEOL EX-270, and Varian Mercury-200BB spectrometers at room temperature in solvents given.

Chemical shifts were expressed in parts per million (ppm) and reported either relative to an internal tetramethylsilane standard (TMS $\delta = 0.0$) or relative to solvent peaks ($CDCl_3$ $\delta = 7.2$, DMSO-d_6 $\delta = 2.5$) for ^{1}H and ($CDCl_3$ $\delta = 77.0$) for ^{13}C. Multiplicities are denoted as follows: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, ddd = double double doublet, m = multiplet, br = broad, apt = apparently. ^{13}C signals were assigned with the aid of DEPT. Coupling constants (*J*) were reported in Hertz (Hz).

*1,2,3,4-Tetra-O-acetyl-β-D-glucopyranuronic acetic anhydride (**1**) [25]*

D-Glucuronic acid (2 g, 10.30 mmol) was suspended in acetic anhydride (30 mL) and stirred at 0 C. Iodine (140 mg, 0.55 mmol) was added and the red solution was left to stir for 30 min at 0°C and a further 2 h at room temperature. Acetic anhydride was mostly removed *in vacuo* and the formed solid was taken up in methylene chloride (50 mL), washed with 1 M $Na_2S_2O_3$ (2 × 30 mL), dried over anhydrous Na_2SO_4, filtered and evaporated to afford the title compound as a white solid (4.08 g, 98%). ^{1}H-NMR (270 MHz, $CDCl_3$): δ 5.80 (d, 1H, $J_{1\text{-}2} = 6.9$ Hz, H-1), 5.32 (m, 2H, H-3 and H-4 overlapping), 5.12 (apt t, 1H, H-2), 4.31 (d, 1H, $J_{5\text{-}4} = 8.7$ Hz, H-5), 2.28 (s, 3H, COCOOCH_3), 2.13 (s, 3H, COCH_3), 2.05 (s, 6H, 2 COCH_3), 2.04 (s, 3H, COCH_3).

*1,2,3,4-Tetra-O-acetyl-β-D-glucopyranuronic acid (**2**) [25]*

1,2,3,4-Tetra-*O*-acetyl-β-D-glucuronic acetic anhydride (**1**, 4.08 g, 10.09 mmol) was dissolved in water and THF (90 mL, 1:2) and stirred overnight. The solution was concentrated and the product was extracted into methylene chloride (3 × 30 mL), the combined organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to yield the title compound as white foam (3.61 g, 99%). ^{1}H-NMR (270 MHz, DMSO-d_6): δ 6.00 (d, 1H, $J_{1\text{-}2} = 8.1$ Hz, H-1), 5.48 (apt t, 1H, H-3), 5.05 (apt t, 1H, H-4), 4.95 (apt t, 1H, H-2), 4.52 (d, 1H, $J_{5\text{-}4} = 8.2$ Hz), 3.44 (br s, 1H, COO*H*), 2.08 (s, 3H, COCH_3), 2.01 (s, 3H, COCH_3), 1.97 (s, 6H, 2 COCH_3).

General procedure for preparation of N-substituted-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide

Triethylamine (1 equiv.) was added to a stirred solution of 1,2,3,4-*O*-tetra-β-D-glucuronic acid (**2**) and ethyl chloroformate (1 equiv.) in methylene chloride (5 mL/0.1 g) cooled to −20°C. After 15 min, the amine (1 equiv.) was added, and the mixture was allowed to warm to −5°C over a period of 1 h. The reaction was then diluted with methylene chloride, transferred to a separating funnel and washed with 1 M HCl, deionized water, saturated $NaHCO_3$, deionized water again, dried over anhydrous Na_2SO_4 and filtered. The solvent was removed and the residue was purified by slow column chromatography (EtOAc/petroleum ether) to afford the title compounds.

*N-Benzyl-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**3**)*

White solid; (46%); mp 97–100°C; $R_f = 0.5$ (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.24–7.17 (m, 5H, aromatic H), 7.05 (t, 1H, N*H*), 6.21 (s, 1H, H-1), 6.18 (d, 1H, H-4), 5.17 (m, 1H, H-3), 5.04 (br s, 1H, H-2), 4.39 (ddd, 2H, CH_2), 1.99, 1.98, 1.97 (3s, 9H, 3 COCH_3); ^{13}C NMR (50 MHz, $CDCl_3$): δ 170.0, 169.6, 169.1 (3 *C*OCH$_3$), 160.6 (*C*ONH), 144.8 (C-5), 137.9 (aromatic *C*), 129.2 (2 × aromatic *C*H), 128.4 (2 × aromatic *C*H), 128.1 (aromatic *C*H), 103.8 (C-4), 88.7 (C-1), 67.5 (C-2), 64.1 (C-3), 43.9 (*C*H$_2$), 21.2, 21.1 (3 CO*C*H$_3$). Anal. calcd. for $C_{19}H_{21}NO_8$ (391.3): C, 58.31; H, 5.41; N, 3.58. Found: C, 58.51; H, 5.49; N, 3.68.

*N-Benzyl-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**4**)*

White solid; (15%); mp 115–117°C; $R_f = 0.50$ (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.67 (t, 1H, N*H*), 7.26–7.17 (m, 5H, aromatic H), 5.94 (d, 1H, $J_{1\text{-}2} = 8.4$ Hz, H-1), 5.40 (apt t, 1H, H-3), 5.12 (apt t, 1H, H-4), 4.95 (apt t, 1H, H-2), 4.33 (d, 1H, $J_{5\text{-}4} = 10.0$ Hz, H-5), 4.20 (ddd, 2H, CH_2) 2.03, 1.97, 1.91, 1.83 (4s, 12H, 4 COCH_3); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 169.8, 169.6, 169.2, 168.7 (4 *C*OCH$_3$), 165.8 (*C*ONH), 137.4 (aromatic *C*), 128.7 (2 × aromatic *C*H), 127.8 (2 × aromatic *C*H), 127.6 (aromatic *C*H), 91.2 (C-1), 72.9, 71.9, 70.1, 68.9 (C-2–C-5), 42.9 (CH$_2$), 20.6, 20.5 (4 CO*C*H$_3$). Anal. calcd. for $C_{21}H_{25}NO_{10}$ (451.4): C, 55.87; H, 5.58; N, 3.10. Found: C, 55.67; H, 5.45; N, 3.39.

*N-Allyl-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**5**)*

Colorless oil; (45%); $R_f = 0.44$ (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.67 (t, 1H, N*H*), 6.30 (d, 1H, $J_{1\text{-}2} = 3.1$ Hz, H-1), 6.25 (d, 1H, H-4), 5.85 (m, 1H, C*H*=CH$_2$), 5.26 (m, 1H, H-3), 5.22–5.15 (m, 2H, CH=C*H*$_2$), 5.14 (br s, 1H, H-2), 3.96 (m, 2H, O*C*H$_2$–CH=CH$_2$), 2.13, 2.12, 2.09 (3s, 9H, 3 COCH_3); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 169.6, 169.1, 168.7 (3 *C*OCH$_3$), 160.0 (*C*ONH), 144.2 (C-5), 133.2 (*C*H=CH$_2$), 116.9 (CH=*C*H$_2$), 103.1 (C-4), 88.2 (C-1), 67.0 (C-2), 63.5 (C-3), 41.7 (O*C*H$_2$–CH=CH$_2$), 20.7, 20.6 (3 CO*C*H$_3$). Anal. calcd. for $C_{15}H_{19}NO_8$ (341.3): C, 52.78; H, 5.61; N, 4.10. Found: C, 52.46; H, 5.43; N, 3.99.

*N-Allyl-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**6**)*

White solid; (18%); mp 182–184°C; $R_f = 0.46$ (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (270 MHz, $CDCl_3$): δ 6.40 (t, 1H, N*H*), 5.84–5.81 (m, 1H, C*H*=CH$_2$), 5.75 (d, 1H, $J_{1\text{-}2} = 7.1$ Hz, H-1), 5.35–5.09 (m, 5H, CH=C*H*$_2$, H-2, H-3 and H-4), 4.10 (d, 1H, $J_{5\text{-}4} = 8.1$ Hz, H-5), 3.86 (m, 2H, O*C*H$_2$–CH=CH$_2$), 2.15, 2.08, 2.05, 2.03 (4s, 12H, 4 COCH_3). Anal. calcd. for $C_{17}H_{23}NO_{10}$ (401.3): C, 50.87; H, 5.78; N, 3.49. Found: C, 50.58; H, 5.49; N, 3.28.

*N-(4-Bromophenyl)-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**7**)*

Yellow oil; (41%); $R_f = 0.74$ (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.2 (s, 1H, N*H*), 7.51 (d, 2H, aromatic H), 7.46 (d, 2H, aromatic H), 6.37 (m, 2H, H-1 and H-4 overlapping), 5.28 (m, 1H, H-3), 5.16 (br s, 1H, H-2), 2.14, 2.12, 2.10 (3s, 9H, 3 COCH_3); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 169.6, 169.2, 168.8 (3 *C*OCH$_3$), 157.9 (*C*ONH), 143.9 (C-5), 135.8 (aromatic *C*-Br), 132.0 (2 × aromatic *C*H), 121.6 (2 × aromatic *C*H), 117.6 (aromatic *C*–NH), 104.3 (C-4), 88.3 (C-1), 67.0 (C-2), 63.4 (C-3), 20.7, 20.6 (3 CO*C*H$_3$). Anal. calcd. for $C_{18}H_{18}BrNO_8$ (456.2): C, 47.39; H, 3.98; Br, 17.51; N, 3.07. Found: C, 47.71; H, 3.78; Br, 17.46; N, 3.24.

*N-(4-Bromophenyl)-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**8**)*

White solid; (18%); mp 173°C (dec.); $R_f = 0.52$ (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (270 MHz, DMSO-d_6): δ 10.30 (s, 1H, N*H*), 7.52

(br s, 4H, aromatic H), 6.06 (d, 1H, $J_{1\text{-}2}$ = 8.4 Hz, H-1), 5.53 (apt t, 1H, H-4), 5.26 (apt t, 1H, H-3), 5.08 (apt t, 1H, H-2), 4.46 (d, 1H, $J_{5\text{-}4}$ = 9.7 Hz, H-5), 2.09, 2.03, 1.97, 1.92 (4s, 12H, 4 COCH_3). Anal. calcd. for $C_{20}H_{22}BrNO_{10}$ (516.3): C, 46.53; H, 4.29; Br, 15.48; N, 2.71. Found: C, 46.41; H, 4.51; Br, 15.62; N, 2.79.

*N-(2-Chloro-4-nitrophenyl)-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**9**)*

Yellow oil; (43%); R_f = 0.85 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 9.15 (s, 1H, N*H*), 8.74 (d, 1H, aromatic H), 8.33 (d, 1H, aromatic H), 8.20 (dd, 1H, aromatic H), 6.44 (d, 1H, $J_{1\text{-}2}$ = 4.6 Hz, H-1), 6.41 (dd, 1H, H-4), 5.32 (m, 1H, H-3), 5.20 (br s, 1H, H-2), 2.15, 2.14, 2.12 (3s, 9H, 3 COCH_3). Anal. calcd. for $C_{18}H_{17}ClN_2O_{10}$ (456.8): C, 47.33; H, 3.75; Cl, 7.76 N, 6.13. Found: C, 47.50; H, 3.52; Cl, 7.49; N, 6.22.

*N-(2-Chloro-4-nitrophenyl)-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**10**)*

White solid; (11%); mp 169–170°C; R_f = 0.25 (EtOAc/petroleum ether, 1:3); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.90 (s, 1H, N*H*), 8.52 (d, 1H, aromatic H), 8.29 (s, 1H, aromatic H), 8.15 (m, 1H, aromatic H), 5.84 (d, 1H, $J_{1\text{-}2}$ = 7.5 Hz, H-1), 5.35 (m, 2H, H-3 and H-4 overlapping), 5.18 (apt t, 1H, H-2), 4.28 (d, 1H, $J_{5\text{-}4}$ = 9.8 Hz, H-5), 2.15, 2.10, 2.06, 2.02 (4s, 12H, 4 COCH_3). Anal. calcd. for $C_{20}H_{21}ClN_2O_{12}$ (516.8): C, 46.48; H, 4.10; Cl, 6.86 N, 5.42. Found: C, 46.59; H, 4.01; Cl, 6.58; N, 5.61.

*N-(Thiazol-2-yl)-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**11**)*

White solid; (43%); mp 103–104°C; R_f = 0.5 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.44 (d, 1H, aromatic H), 6.98 (d, 1H, aromatic H), 6.34–6.32 (m, 2H, H-1 and H-4 overlapping), 5.22 (m, 1H, H-3), 5.12 (br s, 1H, H-2), 2.05, 2.04, 2.01 (3s, 9H, 3 COCH_3); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 170.0, 169.5, 168.8 (3 *C*OCH$_3$), 158.1 (aromatic *C*), 157.5 (*C*ONH), 143.6 (C-5), 138.4 (aromatic *C*), 115.0 (aromatic *C*), 106.1 (C-4), 88.8 (C-1), 67.3 (C-2), 63.8 (C-3), 21.2, 21.1 (3 *C*OCH$_3$). Anal. calcd. for $C_{15}H_{16}N_2O_8S$ (384.3): C, 46.87; H, 4.20; N, 7.29; S, 8.34. Found: C, 46.68; H, 4.12; N, 7.14; S, 8.56.

*N-(Thiazol-2-yl)-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**12**)*

White solid; (10%); mp 178–180°C; R_f = 0.37 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (270 MHz, DMSO-d_6): δ 12.49 (s, 1H, N*H*), 7.50 (d, 1H, aromatic H), 7.31 (d, 1H, aromatic H), 6.08 (d, 1H, $J_{1\text{-}2}$ = 8.1 Hz, H-1), 5.55 (apt t, 1H, H-4), 5.34 (apt t, 1H, H-3), 5.12 (apt t, 1H, H-2), 4.61 (d, 1H, $J_{5\text{-}4}$ = 8.9 Hz, H-5), 2.08, 2.03, 1.97, 1.93 (4s, 12H, 4 COCH_3). Anal. calcd. for $C_{17}H_{20}N_2O_{10}S$ (444.4): C, 45.94; H, 4.54; N, 6.30; S, 7.22. Found: C, 45.70; H, 4.81; N, 6.58; S, 6.98.

*N-(1H-Benzimidazol-2-yl)-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**13**)*

Yellow oil; (39%); R_f = 0.35 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.42 (m, 2H, aromatic H), 7.15 (m, 2H, aromatic H), 6.35 (d, 1H, $J_{1\text{-}2}$ = 4.6 Hz, H-1), 6.27 (d, 1H, H-4), 5.23 (m, 1H, H-3), 5.12 (br s, 1H, H-2), 2.06, 2.02, 2.00 (3s, 9H, 3 COCH_3). Anal. calcd. for $C_{19}H_{19}N_3O_8$ (417.3): C, 54.68; H, 4.49; N, 10.07. Found: C, 54.78; H, 4.41; N, 9.96.

*N-(1H-Benzimidazol-2-yl)-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**14**)*

White solid; (10%); mp 142–144°C; R_f = 0.29 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (270 MHz, DMSO-d_6): δ 7.50 (s, 2H, aromatic H), 7.23 (s, 2H, aromatic H), 6.09 (d, 1H, $J_{1\text{-}2}$ = 9.7 Hz, H-1), 5.56 (apt t, 1H, H-4), 5.35 (apt t, 1H, H-3), 5.10 (apt t, 1H, H-2), 4.65 (d, 1H, $J_{5\text{-}4}$ = 11.5 Hz, H-5), 2.10, 2.04, 1.98, 1.96 (4s, 12H, 4 COCH_3). Anal. calcd. for $C_{21}H_{23}N_3O_{10}$ (477.4): C, 52.83; H, 4.86; N, 8.80. Found: C, 52.66; H, 5.02; N, 8.65.

*Ethyl 1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucpyranuronate (**16**)*

Colorless oil; (49%); R_f = 0.29 (EtOAc/petroleum ether, 1:3); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 6.41 (d, 1H, $J_{1\text{-}2}$ = 2.3 Hz, H-1), 6.27 (dd, 1H, H-4), 5.23 (m, 1H, H-3), 5.15 (br s, 1H, H-2), 4.31 (q, 2H, CH_2), 2.12, 2.11, 2.10 (3s, 9H, 3 COCH_3), 1.34 (t, 3H, CH_3); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 169.7, 169.1, 168.4 (3 *C*OCH$_3$), 161.2 (*C*OOEt), 143.2 (C-5), 106.6 (C-4), 88.5 (C-1), 66.7 (C-2), 63.6 (C-3), 62.0 (OCH_2CH$_3$), 20.8, 20.7 (3 COCH_3), 14.13 (OCH$_2$CH_3). Anal. calcd. for $C_{14}H_{18}O_9$ (330.3): C, 50.91; H, 5.49. Found: C, 50.76; H, 5.14.

*N-(Pyrazin-2-yl)-1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronamide (**17**)*

To a solution of 1,2,3,4-tetra-O-acetyl-β-D-glucuronic acetic anhydride (**1**, 0.5 g, 1.23 mmol) in dry methylene chloride (30 mL) and under argon, 2-aminopyrazine (0.118 g, 1.23 mmol) was added and the reaction mixture was stirred overnight at room temperature. The mixture was then diluted with methylene chloride, transferred to a separating funnel and washed with 1 M HCl (2 × 30 mL), saturated $NaHCO_3$ (2 × 30 mL), deionized water (2 × 30 mL), dried over anhydrous Na_2SO_4 and filtered. The solvent was then removed and the residue was purified by column chromatography (EtOAc/petroleum ether, 1:3) to afford the title compound as a white solid (0.23 mg, 42%); mp 108–110°C; R_f = 0.37 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (270 MHz, $CDCl_3$): δ 9.40 (s, 1H, aromatic H), 8.61 (s, 1H, N*H*), 8.38 (d, 1H, aromatic H), 8.27 (d, 1H, aromatic H), 5.82 (d, 1H, $J_{1\text{-}2}$ = 8.1 Hz, H-1), 5.32 (m, 2H, H-3 and H-4 overlapping), 5.15 (apt t, 1H, H-2), 4.24 (d, 1H, $J_{5\text{-}4}$ = 8.8 Hz, H-5), 2.15, 2.10, 2.05, 2.02 (4s, 12H, 4 COCH_3). Anal. calcd. for $C_{18}H_{21}N_3O_{10}$ (439.3): C, 49.20; H, 4.82; N, 9.56. Found: C, 49.41; H, 4.77; N, 9.46.

*N-(Pyrazin-2-yl)-1,2,3-tri-O-acetyl-Δ^{4,5}-β-D-glucopyranuronamide (**15**)*

DBU (0.05 mL, 0.33 mmol) was added to a stirred solution of *N*-(pyrazin-2-yl)-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranuronamide (**9**, 0.1 g, 0.23 mmol) in dry methylene chloride (5 mL) cooled to 0°C. After stirring for 30 min at 0°C, the solvent was removed *in vacuo* and the dark brown residue was purified by column chromatography (EtOAc/petroleum ether, 1:3) to afford the title compound as a colorless oil (73 mg, 81%); R_f = 0.38 (EtOAc/petroleum ether, 1:1); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 9.51 (s, 1H, aromatic H), 8.77 (s,1H, N*H*), 8.33 (d, 1H, aromatic H), 8.25 (s, 1H, aromatic H), 6.36 (m, 2H, H-1 and H-4 overlapping), 5.26 (m, 1H, H-3), 5.14 (br s, 1H, H-2), 2.09, 2.07, 2.05 (3s, 9H, 3 COCH_3); ^{13}C-NMR (50 MHz, $CDCl_3$): δ 169.5, 169.1, 168.5 (3 *C*OCH$_3$), 158.2 (*C*ONH), 147.2 (aromatic *C*), 143.6 (C-5), 142.3 (aromatic *C*H), 140.8 (aromatic *C*H), 137.1 (aromatic *C*H), 105.2 (C-4), 88.3 (C-1), 66.8 (C-2), 63.3 (C-3), 20.7, 20.6 (3 COCH$_3$). Anal. calcd. for $C_{16}H_{17}N_3O_8$ (379.3): C, 50.66; H, 4.52; N, 11.08. Found: C, 50.39; H, 4.75; N, 10.96.

Biological evaluation

Assay for cytotoxic activity: human cell lines

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. The human tumor cytotoxicities were determined following protocols established by NCI [35]. TK-10, MCF-7, UACC-62 cell lines were cultured in RPMI 1640 medium (BioWhittaker®) containing 20% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, the optimal plating densities of each cell line was determined (15×10^3, 5×10^3 and 100×10^3 cells/well for TK-10, MCF-7 and UACC-62, respectively) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by the sulphorhodamine B (SRB) assay.

Testing procedure and data processing

The sulphorhodamine B (SRB) assay was used in this study to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the SRB dye. For the assay, cells were detached with 0.1% trypsin-EDTA (Sigma Chemical Co.) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final densities of 15×10^4, 5×10^4 and 100×10^4 cells/mL for TK-10, MCF-7 and UACC-62, respectively. One hundred microlitres per well of these cell suspensions was seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h, the cells were treated with the serial concentrations of the synthesized compounds. They were initially dissolved in an amount of 100% DMSO (40 mmol/L) and further diluted in medium to produce five concentrations. One hundred microlitres per well of each concentration was added to the plates to obtain final concentration of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M for the synthesized compounds. The DMSO concentration for the tested dilutions was not greater than 0.25% (v/v), the same as in solvent control wells. The final volume in each well was 200 μL. The plates were incubated for 48 h.

Sulphorhodamine B method

After incubating for 48 h, adherent cell cultures were fixed *in situ* by adding 50 μL of cold 50% (w/v) trichloroacetic acid (TCA) and incubating for 60 min at 4°C. The supernatant was then discarded and the plates washed five times with de-ionized water and dried. One hundred microlitres of SRB solution (0.4% w/v in 1% acetic acid) was added to each microtiter well and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid then the plates were air-dried. Bound stain is solubilised with Tris buffer and the optical densities (OD) were read on an automated spectrophotometric plate reader at a single wavelength of 492 nm. At the end, GI_{50} values (concentrations required to inhibit cell growth by 50%), TGI (concentration resulting in total growth inhibition) and LC_{50} (concentration causing 50% of net cell killing) were calculated according to the previously described protocols [35]. Two or three experiments were carried out for each compound. The data are given as the mean of two or three different assays ±S.E.M.

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