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Synthesis and cytotoxicity of some D-mannose click conjugates with aminobenzoic acid derivatives

Ludmila Hradilová [a,b], Monika Poláková [c,*], Barbora Dvořáková [b], Marián Hajdúch [d], Ladislav Petruš [c]

[a] Department of Organic Chemistry, Palacky University, Tr. 17. listopadu 12, 771 46 Olomouc, Czech Republic
[b] Farmak a.s., Na Vlčinci 3, 77117 Olomouc, Czech Republic
[c] Institute of Chemistry, Center for Glycomics, GLYCOMED, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia
[d] Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and University Hospital in Olomouc, Puškinova 6, 775 20 Olomouc, Czech Republic

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ABSTRACT

Two sets of new conjugates obtained from D-mannose derivatives and *o*-, *m*-, and *p*-substituted benzoic acid esters interconnected through a triazole ring were synthesized by Cu(I) catalyzed azide–alkyne cycloaddition. All synthesized compounds were tested for their in vitro cytotoxic activity against seven cancer cell lines with/without multidrug resistance phenotype as well as non-tumor MRC-5 and BJ fibroblasts. Butyl ester of 4-aminobenzoic acid **6c** showed the highest activity among all tested compounds, however, it was active only against K562 myeloid leukemia cells. *N*-Glycosyltriazole conjugates, both acetylated and nonacetylated at mannose moiety, were almost completely inactive. In contrast, some of the acetylated *O*-glycosyl conjugates showed cytotoxic activity which was cell line dependent and strongly affected by position of benzoic acid substitution as well as a length of its ester alkyl chain; the most potent compound was acetylated mannoside conjugated with octyl ester of *m*-substituted benzoic acid. However, deacetylation resulting in hydrophilicity increase of the glycosides almost completely abolished their cytotoxic potency.

1. Introduction

Cu(I) catalyzed azide–alkyne cycloaddition reaction (CuAAC) became an important tool in organic synthesis due to a regioselective formation of 1,4-disubstituted 1,2,3-triazoles.[1,2] Moreover, the reaction can be performed in polar media including water.[1] It has rapidly attracted attention as a simple and efficient method for the formation of a wide range of compounds wherein two units, one bearing azide and another alkyne function, are linked. Its broad applicability has been documented, for example in the field of biomaterials (hydrogels, microgels),[3–5] polymer bioconjugates,[6–8] drug design,[9,10] and modification of organic or inorganic surfaces (nanoparticles, biosensors),[11–13] etc. In the field of carbohydrate chemistry CuAAC has found a great popularity as well. As overviewed elsewhere,[14–17] simple glycosides, oligo- and polysaccharides, glycomacrocycles, glycopeptides, and glycoclusters have been prepared in this way. Many of 1,4-disubstituted 1,2,3-triazoles as carbohydrate mimetics exhibit diverse biological activities. For example, monosaccharide D-galactose units modified by CuAAC at C-1 or C-6 position were proved to be moderate *Trypanosoma cruzi trans*-sialidase (TcTS) inhibitors functioning as acceptor substrates for TcTS-catalyzed *trans*-sialylation.[18] A series of D-arabinose based glycosyl triazoles with variable hydrophobic groups were synthesized and their biological testing against *Mycobacterium bovis* BCG revealed their low to moderate antimycobacterial activity, with a strong dependence on the nature of the hydrophobic side chain.[19] Several authors reported on a rapid and efficient synthesis of libraries of potential inhibitors of glycogen phosphorylase,[20] sweet almond β-glucosidase,[21] *Escherichia coli* β-galactosidase and bovine liver β-galactosidase,[22] and carbonic anhydrases.[23] Furthermore, a diverse set of conjugates obtained by CuAAC of mannose alkyne (propargyl function at C-1) or azide (at C-6) with organic azides or alkynes, respectively, were utilized to perform a study of the substrate specificity of leishmanian β-1,2-mannosyltransferases.[24] Some of these simple glycosides, 1,4-disubstituted 1,2,3-triazoles, showed also cytotoxic[25,26] and antitumor activity.[27]

Our ongoing research has been focused on a derivatization and modification of aromatic as well as carbohydrate units with intention to obtain compounds with potential biological effects and with this aim many interesting D-mannose based derivatives have been synthesized and evaluated.[28–30] As a continuation of our work we focused on preparation of a series of new conjugates from D-mannose derivatives (propargyl or azide) with counterparts derived from *o*-, *m*-, and *p*-substituted benzoic acid esters by CuAAC.

* Corresponding author. Tel.: +421 259410272; fax: +421 259410222.
E-mail address: Monika.Polakova@savba.sk (M. Poláková).

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Both types of the conjugates, one optionally possessing ester function at aromatic carboxylate and another one bearing amino function at aromatic unit, were investigated and evaluated for their cytotoxic activity against seven tumor and two non-malignant cell lines.

2. Results and discussion

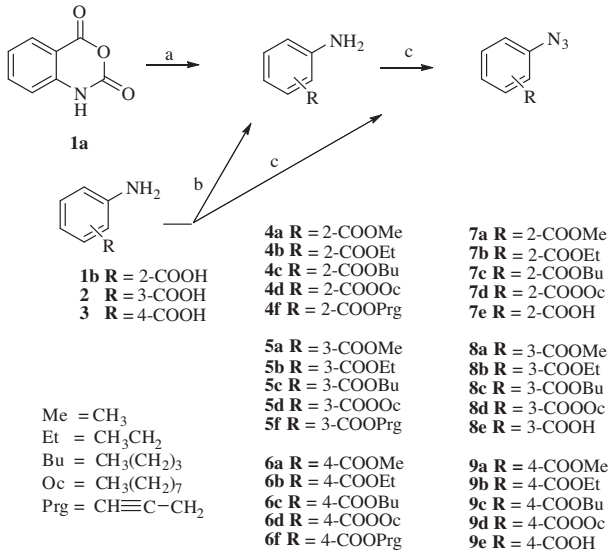
Synthetic routes leading to aromatic units are depicted in Scheme 1

A set of *o*-, *m*-, and *p*-substituted benzoic acid esters with variable length of alkyl ester chain was synthesized. Among them, amino substituted propargyl esters belonged to one type of the building blocks for the click reaction. All other esters comprising free amino group were transformed to azides to get another set of the building blocks with aromatic nature.

ortho Substituted aminobenzoic acid esters (anthranilic acid esters) **4a**–**d**, **4f** were prepared by the reaction of isatoic anhydride **1a** and appropriate alcohol in toluene with sodium hydride as a catalyst.[31] While short esters of *m*- and *p*-aminobenzoic acid, namely methyl, ethyl, and butyl (**5a–c**, **6a**–**c**) were easily obtained by a reaction of acid **2** or **3** in an excess of the corresponding alcohol and thionyl chloride,[32] for the preparation of octyl and propargyl esters (**5d, 5f** and **6d, 6f**) it was necessary to employ a modified methodology, previously applied for the synthesis of salicylic acid esters[33] starting from potassium salt of the acid, which reacted with alkyl halide in DMF.

The esters prepared are known mostly as oily compounds. Their structures were confirmed by HRMS (Table 1,Supplementary data). For propargyl esters **4f**, **5f**, and **6f**, NMR analysis was carried out as well (Table 2, Supplementary data).

Aromatic azides were obtained from the corresponding amines by conventional means through diazonium salts. Generally, this reaction proceeded at low temperature (−10 °C). In the present study, the conditions[34] were modified and the reaction was performed at 5 °C, either in water (when starting from acid) or in acetone (when ester was a starting material). By this way the following new azides **7c**, **7d**, **8c**, **8d**, **9c**, and **9d** (all as oily material) were prepared in addition to known derivatives **7a**, **7b**, **8a**, **8b**, **9a**, and **9b**.



Scheme 1. Synthesis of benzoic acid derivatives bearing either azido or propargyl function. Reagents and conditions: (a) alcohol, NaH, toluene, reflux, 2 h, **4a**(91%), **4b**(89%), **4c**(56%), **4d**(48%), **4f**(82%); (b) **5a**–**c**,**6a**–**c**: alcohol and thionylchloride, 30–65 °C, 4–10 h; **5d**,**6d**: K_2CO_3, octyl chloride, DMF, 70–80 °C, 2 h; **5f**,**6f**: K_2CO_3, propargyl bromide, DMF, rt, 1 h, **5a**(91%), **5b**(88%), **5c**(81%), **5d**(51%), **5f**(89%), **6a**(91%), **6b**(83%), **6c**(76%), **6d**(48%), 6f(68%); (c) (1) $NaNO_2$, HCl, acetone, (2) NaN_3.

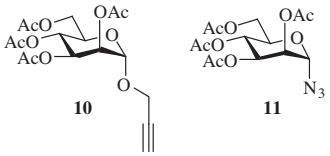


Figure 1. Structure of sugar units.

All azides were used directly in the next step without purification and characterization since their synthesis through diazonium salts afforded sufficiently clean products.[31,34]

D-Mannose based units were synthesized as counterparts of aromatic building blocks. In this case the alkyne or azido function was introduced at the α-anomeric position. These mannose derivatives (Figure 1), 2-propynyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside **10**[35] and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl azide **11**[36] were prepared by Lewis acid catalyzed treatment of peracetylated mannose with the corresponding acceptor.

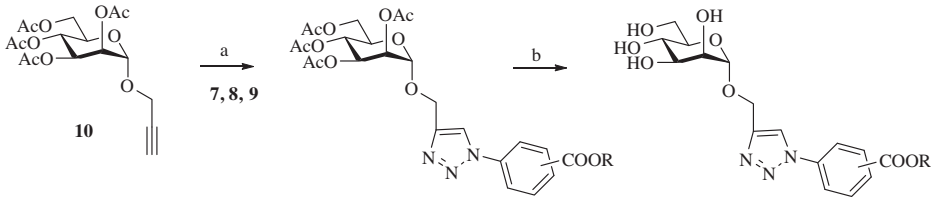
During past years various procedures using either ascorbic acid or its sodium salt in combination with Cu salts were developed and described as efficient protocols for the CuAAC.[37] Having prepared a large set of appropriate building blocks, coupling reaction between aromatic and mannose units was promoted by copper(II) sulfate and ascorbic acid in DMF/H_2O. By this way two sets of target new conjugates were prepared in moderate to good yields. Set (A) consists of *O*-glycosyl conjugates **12**–**14**, obtained by coupling propargyl mannoside **10** with the set of *o*-, *m*-, or *p*-azidobenzoic acid esters (Scheme 2). Set (B) consists of *N*-glycosyl conjugates **18**–**20**, generated by the reaction of mannose azide **11** with propargyl ester of *o*-, *m*-, or *p*-aminobenzoic acid (Scheme 3). Their structures were identified based on the characteristic signals in the ^1H NMR spectrum: the olefinic proton belonging to the 1,2,3-triazole moiety appeared as a singlet at δ 7.87–8.18 for set (A) and at δ 7.86–7.87 for set (B) of the conjugates, depending on the position of aromatic unit substitution.

Final removal of acetyl protecting groups appeared as most problematic. The yields of the desired target compounds strongly depended on the quantity of potassium carbonate, which was found to be the best reagent. For deprotection of all derivatives bearing ethyl, butyl, and octyl ester function maximum required amount was 0.1 equiv K_2CO_3 since side reactions like re-esterification or breakdown of the molecule were observed if the amount of the base was higher. All other acetylated derivatives were deprotected using 0.2 equiv of K_2CO_3. After the deprotection, the signal of olefinic proton belonging to 1,2,3-triazole moiety was downfielded to $\delta \approx 8.24$–9.00 and $\delta \approx 8.24$–8.27 for compounds **15**–**17** in set (A) and **21**–**23** in set (B), respectively. Moreover, the signals of the ester chain were observed at expected fields, thus confirming the structures of deprotected conjugates.

All procedures were optimized. The structures of all new compounds were confirmed by NMR spectroscopy and mass spectrometry.

2.1. Biological activity

All synthesized compounds, namely esters **4–6**, acetylated conjugates **12–14, 18–20**, and deacetylated conjugates **15–17, 21–23** were tested under in vitro MTT test[38] against cancer cell lines CEM (T-lymphoblastic leukemia), K562 (myeloid leukemia), their drug resistant counterparts CEM-DNR-bulk (overexpressing the multidrug resistant protein 1) and K562-TAX (overexpressing the P-glycoprotein), and A549 (lung adenocarcinoma), HCT116 (colorectal cancer with wildtype p53), HCT116p53 −/− (colorectal cancer with deleted p53), and non-malignant BJ and MRC-5 fibroblasts.



12a R = 2-COOMe
12b R = 2-COOEt
12c R = 2-COOBu
12d R = 2-COOOc
12e R = 2-COOH

13a R = 3-COOMe
13b R = 3-COOEt
13c R = 3-COOBu
13d R = 3-COOOc
13e R = 3-COOH

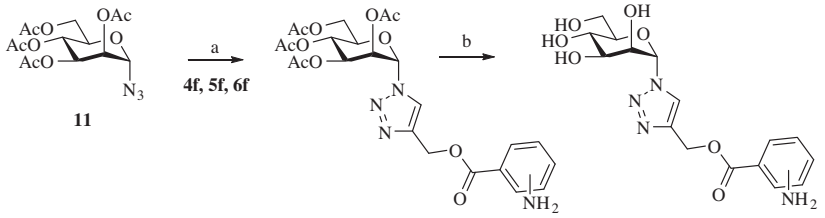
14a R = 4-COOMe
14b R = 4-COOEt
14c R = 4-COOBu
14d R = 4-COOOc
14e R = 4-COOH

15a R = 2-COOMe
15b R = 2-COOEt
15c R = 2-COOBu
15d R = 2-COOOc
15e R = 2-COOH

16a R = 3-COOMe
16b R = 3-COOEt
16c R = 3-COOBu
16d R = 3-COOOc
16e R = 3-COOH

17a R = 4-COOMe
17b R = 4-COOEt
17c R = 4-COOBu
17d R = 4-COOOc
17e R = 4-COOH

Scheme 2. Synthesis of the set (A): *O*-glycosyl conjugates. Reagents and conditions: (a) ascorbic acid, $CuSO_4 \cdot 5H_2O$, DMF, DMF/H_2O, **12a**(65%), **12b**(58%), **12c**(55%), **12d**(58%), **12e**(74%), **13a**(62%), **13b**(55%), **13c**(55%), **13d**(56%), **13e**(70%), **14a**(56%), **14b**(46%), **14c**(44%), **14d**(45%), **14e**(64%); (b) K_2CO_3, MeOH, **15a**(76%), **15b**(73%), **15c**(63%), **15d**(47%), **15e**(90%), **16a**(84%), **16b**(69%), **16c**(68%), **16d**(45%), **16e**(80%), **17a**(67%), **17b**(69%), **17c**(62%), **17d**(49%), **17e**(76%).



18 2-NH_2
19 3-NH_2
20 4-NH_2

21 2-NH_2
22 3-NH_2
23 4-NH_2

Scheme 3. Synthesis of the set (B): *N*-glycosyl conjugates. Reagents and conditions: (a) ascorbic acid, $CuSO_4 \cdot 5H_2O$, DMF/H_2O, rt, **18**(64%), **19**(74%), **20**(70%); (b) K_2CO_3, MeOH, rt, **21**(53%), **22**(49%), **23**(44%).

Table 1
Cytotoxicity results (IC_{50} (μM))[a]

	CEM	CEM-DNR	K562	K562-TAX	A549	HCT116	HCT116 p53−/−	BJ	MRC-5
Doxorubicin	0.01	7.4	0.4	6.1	0.2	0.5	0.8	7.2	5.3
4d	93.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5d	72.3	79.2	39.8	n.a.	n.a.	61.5	59.1	n.a.	n.a.
6a	n.a.	n.a.	76.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6c	n.a.	n.a.	19.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6d	58.7	55.7	51.8	53.2	61.3	65.5	61.7	n.a.	n.a.
6f	n.a.	n.a.	69.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12d	23.8	40.5	71.6	31.9	67.1	51.4	46.7	n.a.	n.a.
13c	72.1	n.a.	n.a.	n.a.	n.a.	79.2	n.a.	n.a.	n.a.
13d	31.0	34.4	31.7	48.4	23.5	45.2	36.5	n.a.	64.2
14c	77.2	n.a.	n.a.	n.a.	75.7	74.3	71.9	69.5	76.1
14d	74.1	66.8	n.a.	72.6	45.9	59.6	68.8	n.a.	n.a.
15d	40.4	n.a.	43.5	n.a.	74.1	n.a.	n.a.	n.a.	n.a.
16d	75.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
17d	72.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
22	n.a.	77.7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

[a] Average values for IC_{50} (μM) from 3 to 4 independent experiments with the standard deviation ranging from exceeding 10–25% of the average values. n.a.—no cytotoxic activity (e.g., >250 μM). Synthesized compounds not given in the table showed no cytotoxic activity. Doxorubicin—a reference cytostaticum.

Among anthranilic acid esters **4a–f**, only octyl ester **4d** exhibited some activity (IC_{50} 93.2 μM toward cell line CEM), otherwise they were inactive in all cell lines. Among methyl and ethyl esters of 3- and 4-aminobenzoic acids, **5a**, **5b**, and **6b** were inactive against all cell lines and **6a** was an exception being moderately active only in line K562 (IC_{50} 76.5 μM). Similarly, other esters of

3-aminobenzoic acid and 4-aminobenzoic acid exhibited the highest cytotoxic activity against myeloid leukemia K562. Since this cell line bears specific oncogenic translocation of BCR and ABL genes, one could hypothesize that possible molecular target for the active compound is bcr–abl fusion protein. It was also inhibited by butyl ester **6c** (IC_{50} 19.4 μM, the highest inhibition observed in this study), which was otherwise inactive. In contrast, octyl ester **6d** was the only ester active against all tumor cell lines (IC_{50} in the range 50–80 μM) except of normal BJ and MRC-5 fibroblasts.

For conjugates in set (A) the coupling led to an increase in cytotoxic activity in the case of octyl ester in *ortho* position of aromatic unit (cf. IC_{50} 93.2 μM of **4d** and 23.8 μM of **12d**, CEM lymphoblasts). In addition, a relatively high inhibition activity was also retained after removal of acetyl groups (**15d**, IC_{50} 40.4 μM). It is interesting to note that acetylated conjugate **12d** was active against more drug-sensitive as well as drug-resistant cancer cell lines and with higher potency in comparison with the deprotected counterpart **15d**. The only exception was cell line K562 which was more susceptive toward the latter compound **15d** (Table 1).

The substitution in *meta* position of aromatic unit of the conjugate, in particular octyl ester, was generally observed to be the most preferred to achieve the highest activity. The acetylated conjugate **13d** exhibited activity against the widest spectrum of cell lines among all tested compounds. It affected all drug sensitive and drug resistant cell lines, and interestingly also one normal fibroblast cell line (MRC-5). However, removal of acetyl groups from mannose unit led, except for one line (CEM), to complete loss of activity.

The conjugates with *para* substitution at aromatic unit exhibited the weakest cytotoxic activity. Acetylated octyl ester **14d** was the most active compound among this group of conjugates, however only weak cytotoxic activity was exhibited against solid tumor cell lines A549 (IC_{50} 45.9 μM) and HCT116 (IC_{50} 59.6 μM). Acetylated butyl ester **14c** also inhibited some cancer lines, however, it was the only compound active on both non-tumor fibroblast cell lines included in this study, thus suggesting general cytotoxicity of the derivative.

The coupling of azide **11** with propargyl ester of *o*-, *m*-, or *p*-substituted aminobenzoic acid, giving conjugates **18**–**23** of set (B), did not result in biologically potent compounds since among them only **22** exhibited IC_{50} 77.7 μM against CEM-DNR-bulk cells.

3. Conclusion

Substituted benzoic acid esters and D-mannose based new conjugates derived thereof were synthesized and tested in vitro against tumor and non-malignant fibroblast cell lines. The most important factors affecting their cytotoxic activity were the carboxyl ester chain length of aromatic unit and a position of this substitution. Of non-mannosylated aminobenzoic acid derivatives maximum cytotoxic activity (IC_{50} 19.8 μM) accompanied with an exclusive selectivity toward drug-sensitive cell line K562 was observed for butyl ester **6c**. Coupling of mannose azide **11** to the esters in the form of *N*-glycosyl conjugates (compounds **18**–**23**) did not lead to an appearance of biological activity. Similarly, synthesis of *O*-glycosyl conjugates prepared by the conjugation of propargyl mannoside **10** with azidobenzoic acids or their methyl, ethyl, and butyl esters, did not result in cytotoxicity. In contrast, octyl esters of the *O*-glycosyl conjugates showed, in general, not only increased cytotoxic activity but also affected a wider spectrum of tumor and normal fibroblast cell lines. Both conclusions are evident for acetylated octyl esters **12d**, **13d**, and **14d**, although significant decrease of IC_{50} as well as narrowing of malignant cell lines influenced was observed after their deacetylation.

It is also noteworthy and may be of practical implications that drug sensitive hematological cancer lines CEM and K562 are the most susceptive not only to the *O*-glycosyl conjugates but also to the non-glycosylated aminobenzoic acid esters.

4. Experimental

4.1. General methods

TLC was performed on plastic plates precoated with silica gel Polygram Sil G/UV254. The spots were visualized by spraying with 10% sulfuric acid in ethanol and heating. Melting points were measured in a Köfler apparatus and are uncorrected. Microanalyses were obtained using a Fisons EA-1108 instrument.Column chromatography was carried out on SilicaFlash P60 (40–63 μm, Silicycle) with distilled solvents (toluene, ethyl acetate, and methanol). Dichloromethane was dried (CaH_2) and distilled before use. All reactions containing sensitive reagents were carried out under argon atmosphere. ^1H NMR and ^{13}C NMR spectra were recorded at 25 °C with a Varian spectrometer (399.90 MHz for ^1H, 100.56 MHz for ^{13}C) in $CDCl_3$, CD_3OD, or DMSO. The ^1H and ^{13}C chemical shifts were referenced to the central signal of the solvent: $CDCl_3$ ($\delta = 7.26$ (^1H), $\delta = 77.0$ (^{13}C)); CD_3OD ($\delta = 3.31$ (^1H), $\delta = 49.1$ (^{13}C)); and DMSO ($\delta = 2.55$ ^1H and $\delta = 39.6$ (^{13}C)). The assignment of resonances in the ^1H and ^{13}C NMR spectra was made by two-dimensional homonuclear and heteronuclear shift correlation experiments. Optical rotations were measured on a Jasco P2000 polarimeter at 20 °C. High resolution mass determination was performed by ESI-MS on a Thermo Scientific Orbitrap Exactive instrument operating in positive or negative mode. All derivatives were freeze-dried immediately after their column chromatography purification and dissolved prior use.

4.2. Chemistry

4.2.1. Synthesis of anthranilic acid esters (4a–d)

Dispersion of 55% sodium hydride in mineral oil (20 mg, 0.46 mmol) was added to a suspension of isatoic anhydride **1a** (1.5 g, 9.20 mmol) in a corresponding alcohol (60 mL); octanol (1.3 g, 9.98 mmol) in toluene (50 mL) was used for derivative **4d**. The reaction mixture was heated at reflux until TLC (toluene/EtOAc/formic acid 25:25:1) indicated that the starting isatoic anhydride was consumed. The reaction mixture was concentrated and the residue was stirred with satd $NaHCO_3$ (30 mL) for 15 min and extracted with EtOAc (3 × 30 mL). The organic extracts were pooled, dried (Na_2SO_4), and concentrated to give **4a–d** as an oil and **4f** as a crystal of low melting point. Esters **4a**[39] and **4b**[32] were used without purification, esters **4c**,[32,40a,b] **4d**,[41a,b] and **4f**[42] were purified by column chromatography (toluene/EtOAc 9:1). Analytical data (HRMS and melting points) and yields are in Table 1 (Supplementary data). ^1H and ^{13}C NMR data for **4f** are in Table 2 (Supplementary data).

4.2.2. Synthesis of 3-aminobenzoic acid esters (5a–c) and 4-aminobenzoic acid esters (6a–c)

Acid **2** or **3** (1.5 g, 10.94 mmol) was dissolved in the corresponding alcohol (150 mL) at temperature about 50 °C. Then the solution was cooled to rt and thionyl chloride (15 mL, 206.77 mmol) was added. The reaction mixture was heated at 30 °C (MeOH), 50 °C (EtOH), or 65 °C (BuOH). The reaction course was monitored by TLC (toluene/EtOAc/formic acid 25:25:1). When the starting acid was consumed (4–10 h), water (20 mL) was added. The reaction mixture was stirred for 30 min and concentrated. The resulting residue was stirred with satd $NaHCO_3$ (30 mL) for 45 min and extracted with EtOAc (3 × 30 mL). The organic extracts were

pooled, dried (Na_2SO_4), and concentrated to obtain esters **5a**,[43] **5b**,[32,44] **5c**,[32] **6a**,[45] **6b**,[32,46,47] and **6c**[32] as an oil. All esters were used in the next step without purification.

Analytical data (HRMS) and yields are in Table 1 (Supplementary data). ^1H and ^{13}C NMR data for **5f** and **6f** are in Table 2 (Supplementary data).

4.2.3. Alkylation of 3-aminobenzoic acid (2) or 4-aminobenzoic acid (3)

To a solution of acid **2** or **3** (1.5 g, 10.94 mmol) in DMF (15 mL) was added K_2CO_3 (1.51 g, 10.93 mmol) and the solution was heated under reflux for 5 min. Then the reaction mixture was cooled to rt and the corresponding alkyl halide (10.9 mmol) was added. The reaction mixture was heated to 70–80 °C for 2 h, then cooled to rt, poured into water (150 mL) and extracted with EtOAc (3 × 60 mL). Pooled organic layers were washed with water, dried (Na_2SO_4), and concentrated to give esters **5d**[48] and **6d**[49] as an oil and **5f**,[50] **6f**,[51] as a solid. All esters were used directly in the next step without purification.

Analytical data (HRMS and melting points) and yields are in Table 1 (Supplementary data). ^1H and ^{13}C NMR data for **5f** and **6f** are in Table 2 (Supplementary data).

4.2.4. Synthesis of azido derivatives (7a–e, 8a–e) and (9a–e)

Acid **1b**, **2**, or **3** (7.29 mmol), or ester **4, 5**, or **6** (7.29 mmol) was dissolved in 15 mL of water or acetone, respectively. The solution was cooled to 0 °C and concentrated hydrochloric acid (2.5 mL) was added dropwise. A solution of sodium nitrite (0.5 g, 7.25 mmol) in water (3 mL) was added at a temperature below 0 °C and the reaction mixture was stirred for 10 min at this temperature. Then solution of sodium acetate (0.41 g, 5.0 mmol) in water (5 mL) was added to the reaction mixture followed by an addition of solution of sodium azide (0.47 g, 7.23 mmol) in water (3 mL) at temperature below 5 °C. When no starting material was detected by TLC (toluene/EtOAc/formic acid 25:25:1), the reaction mixture was poured into ice cold water (15 mL). The precipitated solid azides **7e**,[52a,b] **8e**,[53] **9a**,[52b,54a,b] and **9e**[54b,55a,b] were filtered off and washed with water. Oily azides **7a**,[52b,56] **7b**,[57] **7c**, **7d**, **8a**,[58] **8b**,[54b,59a,b] **8c**, **8d**, **9b**,[60] **9c**, and **9d** were extracted with EtOAc (3 × 20 mL), organic extracts were pooled, dried (Na_2SO_4), and concentrated. All azides were used directly in the next step without analysis or further purification.

4.2.5. Synthesis of conjugates (12–14) and (18–20)

To a solution of azide **7**, **8**, **9**, or **11** (5.69 mmol) and propargyl derivative **10**, **4f**, **5f**, or **6f** (5.69 mmol), respectively, in DMF (15 mL), $CuSO_4 \cdot 5H_2O$ (0.4 g, 1.60 mmol) solution in water (4 mL) was added followed by a solution of ascorbic acid (1.0 g, 5.68 mmol) in water (8 mL). The reaction mixture was stirred at rt for about 45 min when TLC (toluene/EtOAc/formic acid 25:25:1) indicated consumption of starting material. The reaction mixture was poured into water (150 mL) and the product was extracted with EtOAc (3 × 60 mL). To the organic phase charcoal was added and the former was filtered and concentrated. The crude product was purified by column chromatography (toluene/EtOAc 9:1).

Analytical data (HRMS, optical rotation, and elemental analysis) and yields are in Table 3 (Supplementary data). ^1H and ^{13}C NMR data are in Tables 4–7 (Supplementary data).

4.2.6. Removal of acetyl protective groups. Synthesis of conjugates (15–17) and (21–23)

A: Compounds **12b–d, 13b–d, and 14b–d** (0.3 mmol), were dissolved in methanol (5 mL). Potassium carbonate (4.1 mg, 0.03 mmol) was added thereto.

B: Compounds **12a**, **12e**, **13a**, **13e**, **14a**, **14e, 18–20** (0.3 mmol) were dissolved in methanol (5 mL). Potassium carbonate (8.3 mg, 0.06 mmol) was added thereto.

Then the reaction mixture was stirred at rt until disappearance of starting material (0.75–3 h) and concentrated. The crude product was purified by column chromatography (EtOAc/methanol 9:1) to give the target compounds **15–17** and **21–23**. (Purification must be quick because of limited compound stability).

Analytical data (HRMS, optical rotation, and elemental analysis) and yields are in Table 3 (Supplementary data). ^1H and ^{13}C NMR data are in Tables 8–11 (Supplementary data).

4.3. Biological activity

4.3.1. Cell lines

All cells were purchased from the American Tissue Culture Collection (ATCC), unless otherwise indicated. The daunorubicin-resistant subline of CEM cells (CEM-DNR bulk) and paclitaxel-resistant subline K562-tax were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicine or paclitaxel, respectively.[61] The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

4.3.2. Cytotoxic MTT assay[38,61]

The cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500–30,000 cells/well based on cell growth characteristics). The cells were added by pipette (80 μL) into 96-well microtiter plates. The inoculates were allowed a pre-incubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Fourfold dilutions, in 20 μL aliquots, of the intended test concentration were added to the microtiter plate wells at time zero. All test compound concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in 5% CO_2 at 100% humidity. At the end of the incubation period, the cells were assayed by using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for a further 1–4 h. After this incubation period, the formazan produced was dissolved by the addition of 100 μL/well of 10% aq SDS (pH 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. The tumor cell inhibitory concentration (IC) of each test compound was calculated using the following equation: $IC = (OD_{\text{drug-exposed well}}/\text{mean } OD_{\text{control wells}}) \times 100\%$. The IC_{50} value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose–response curves.

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Supplementary data

Supplementary data (analytical data (^{1}H and ^{13}C NMR chemical shifts, optical rotation, HRMS and elemental analysis of the synthesized compounds) and yields.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.08.001.

References

1. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.
2. Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057–3064.
3. Ossipov, D. A.; Hilborn, J. *Macromolecules* **2006**, *39*, 1709–1718.
4. Crescenzi, V.; Cornelio, L.; DiMeo, C.; Nardecchia, S.; Lamanna, R. *Biomacromolecules* **2007**, *8*, 1844–1850.
5. Duckworth, B. P.; Xu, J.; Taton, T. A.; Guo, A.; Distefano, M. D. *Bioconjug. Chem.* **2006**, *17*, 967–974.
6. Lutz, J. F.; Börner, H. G.; Weichenhan, K. *Macromolecules* **2006**, *39*, 6376–6383.
7. Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046–15047.
8. Deiters, A.; Cropp, T. A.; Summerer, D.; Mukherji, M.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5743–5745.
9. Kolb, H. C.; Sharpless, K. B. *Drug Discovery Today* **2003**, *8*, 1128–1137.
10. Manetsch, R.; Krasinski, A.; Radic, Z.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C. *J. Am. Chem. Soc.* **2004**, *126*, 12809–12818.
11. Devaraj, N. K.; Miller, G. P.; Ebina, W.; Kakaradov, B.; Collman, J. P.; Kool, E. T.; Chidsey, C. E. D. *J. Am. Chem. Soc.* **2005**, *127*, 8600–8601.
12. Decreau, R. A.; Collman, J. P.; Yang, Y.; Yan, Y.; Devaraj, N. K. *J. Org. Chem.* **2007**, *72*, 2794–2802.
13. Lin, P. C.; Ueng, S. H.; Yu, S. C.; Jan, M. D.; Adak, A. K.; Yu, C. C.; Lin, C. C. *Org. Lett.* **2007**, *9*, 2131–2134.
14. Arago-Leoneti, V.; Campo, V. L.; Gomes, A. S.; Field, R. A.; Carvalho, I. *Tetrahedron* **2010**, *66*, 9475–9492.
15. Dedola, S.; Nepogodiev, S. A.; Field, R. A. *Org. Biomol. Chem.* **2007**, *5*, 1006–1017.
16. Dondoni, A. *Chem. Asian J.* **2007**, *2*, 700–708.
17. Santoyo Gonzalez, F.; Hernandez-Mateo, F. *Top. Heterocycl. Chem.* **2007**, *7*, 133–177.
18. Carvalho, P.; Andrade, P.; Campo, V. L.; Guedes, P. M. M.; Si-Costa, R.; Silva, J. S.; Schenkman, S.; Dedola, S.; Hill, L.; Rejzek, M.; Nepogodiev, S. A.; Field, R. A. *Bioorg. Med. Chem.* **2010**, *18*, 2412–2427.
19. Wilkinson, B. L.; Long, H.; Sim, E.; Fairbanks, A. J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6265–6267.
20. Bokor, E.; Somsak, L.; Docsa, T.; Gergely, P. *Bioorg. Med. Chem.* **2010**, *18*, 1171–1180.
21. Dedola, S.; Hughes, D. L.; Nepogodiev, S. A.; Rejzek, M.; Field, R. A. *Carbohydr. Res.* **2010**, *345*, 1123–1134.
22. Rossi, L. L.; Basu, A. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3596–3599.
23. Wilkinson, B. L.; Innocenti, A.; Vullo, D.; Supuran, C. T.; Poulsen, S.-A. *J. Med Chem.* **2008**, *51*, 1945–1953.
24. van der Peet, P.; Gannon, C. T.; Walker, I.; Dinev, Z.; Angelin, M.; Tam, S.; Ralton, J. E.; McConville, M. J.; Williams, S. J. *ChemBioChem* **2006**, *7*, 1384–1391.
25. Manach, C. L.; Baron, A.; Guillot, R.; Vauzeilles, B.; Beau, J. M. *Tetrahedron Lett.* **2011**, *52*, 1462–1465.
26. dos Anjos, J. V.; Neves, R. A. W.; Do Nascimento, S. C.; Srivastava, R. M.; de Melo, S. J.; Sinou, D. *Eur. J. Med. Chem.* **2009**, *4*, 3571–3576.
27. Yu, L.; Wua, Q.-P.; Zhang, Q.-S.; Liu, Y.-H.; Li, Y.-Z.; Zhou, Z.-M. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 240–243.
28. Poláková, M.; Beláňová, M.; Petruš, L.; Mikušová, K. *Carbohydr. Res.* **2010**, *345*, 1339–1347.
29. Poláková, M.; Šesták, S.; Lattová, E.; Petruš, L.; Mucha, J.; Tvaroška, I.; Kóňa, J. *Eur. J. Med. Chem.* **2011**, *46*, 944–952.
30. Poláková, M.; Beláňová, M.; Mikušová, K.; Lattová, E.; Perreault, H. *Bioconjug. Chem.* **2011**, *22*, 289–298.
31. Hradilová, L.; Grepl, M.; Hlaváč, J.; Lyčka, A.; Hradil, P.; *J. Heterocyclic Chem.*, in press.
32. Hosangadi, B. D.; Dave, R. H. *Tetrahedron Lett.* **1996**, *37*, 6375–6378.
33. Ueno, Y.; Jose, J.; Loudet, A.; Perez-Bolivar, C.; Anzenbacher, P.; Burgess, K. *J. Am. Chem. Soc.* **2011**, *133*, 51–55.
34. Pokhodylo, N. T.; Matiychuk, V. S. *J. Heterocyclic Chem.* **2010**, *47*, 415–420.
35. Hasegawa, T.; Numata, M.; Okumura, S.; Kimura, T.; Sakurai, K.; Shinkai, S. *Org. Biomol. Chem.* **2007**, *5*, 2404–2412.
36. Paulsen, H.; Györgydeák, Z.; Friedmann, M. *Chem. Ber.* **1974**, *107*, 1568–1578.
37. Bokor, É.; Koppány, C.; Gonda, Z.; Novák, Z.; Somsák, L. *Carbohydr. Res.* **2012**, *351*, 42–48.
38. Hajdúch, M.; Mihál, V.; Minařík, J.; Faber, E.; Šafářová, M.; Weigl, E.; Antalek, P. *Cytotechnology* **1996**, *19*, 243–245.
39. Lu, W.; Chen, J.; Liu, M.; Ding, J.; Gao, W.; Wu, H. *Org. Lett.* **2011**, *13*, 6114–6117.
40. (a) Pytela, O.; Halama, A. *Coll. Czech. Chem. Comm.* **1996**, *61*, 751–763; (b) Hoechst, Patent FR2361341 DE2636423, 1978.
41. (a) Barry, J.; Bram, G.; Petit, A. *Heterocycles* **1985**, *23*, 875–880; (b) Mobil Oil Corporation Patent US4021470 A1, 1977.
42. Garanti, L.; Molteni, G.; Zecchi, G. *Heterocycles* **1994**, *38*, 291–296.
43. Broch, S.; Heenon, H.; Anizon, F.; Moreau, P.; Debaud, A.-L.; Fogeron, M.-L.; Bonnefoy-Berard, N. *Bioorg. Med. Chem.* **2010**, *18*, 7132–7143.
44. Monguchi, Y.; Maejima, T.; Mori, S.; Maegawa, T.; Sajiki, H. *Chem.-Eur. J.* **2010**, *16*, 7372–7375.
45. Nicolaou, K. C.; Natarajan, S.; Li, H.; Jain, N. F.; Hughes, R.; Solomon, M. E.; Ramanjulu, J. M.; Boddy, C. N. C.; Takayanagi, M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2708–2714.
46. Markiewicz, J. T.; Wiest, O.; Helquist, P. *J. Org. Chem.* **2010**, *75*, 4887–4890.
47. Zafar, A.; Melendez, R.; Geib, S. J.; Hamilton, A. D. *Tetrahedron* **2002**, *58*, 683–690.
48. OJI PAPER CO., LTD. Patent EP1264707 A2, 2002.
49. Blackburn, O. A.; Coe, B. J.; Fielden, J.; Helliwell, M.; McDouall, J. J. W.; Hutchings, M. G. *Inorg. Chem.* **2010**, *49*, 9136–9150.
50. Norvartis Corporation Patent US5856578 A1, 1999.
51. Onopchenko, A.; Sabourin, E. T.; Selwitz, C. M. *J. Org. Chem.* **1979**, *44*, 3671–3674.
52. (a) Shaw, A. Y.; Chen, Y.-R.; Tsai, C.-H. *Synth. Commun.* **2009**, *39*, 2647–2663; (b) Bou-Hamdan, F. R.; Levesque, F.; O'Brien, A. G.; Seeberger, P. H. *Beilstein J. Org. Chem.* **2011**, *7*, 1124–1129.
53. Juwarker, H.; Lenhardt, J. M.; Pham, D. M.; Craig, S. L. *Angew. Chem., Int. Edit.* **2008**, *47*, 3740–3743.
54. (a) Morawietz, J.; Sander, W.; Traeubel, M. *J. Org. Chem.* **1995**, *60*, 6368–6378; (b) Li, Y.; Gao, L.-X.; Han, F.-S. *Chem. Eur. J.* **2010**, *16*, 7969–7972.
55. (a) Wilkening, I.; del Signore, G.; Hackenberger, C. P. R. *Chem. Commun.* **2011**, *47*, 349–351; (b) Grimes, K. D.; Gupte, A.; Aldrich, C. C. *Synthesis* **2010**, *9*, 1441–1448.
56. Purvis, R.; Smalley, R. K.; Suschitzky, H.; Alkhader, M. A. *J. Chem. Soc., Perkin Trans. 1* **1984**, 249–254.
57. Ding, M.-W.; Zeng, G.-P.; Wu, T.-J. *Synth. Commun.* **2000**, *30*, 1599–1604.
58. Kanakarajan, K.; Goodrich, R.; Young, M. J. T.; Soundararajan, S.; Platz, M. S. *J. Am. Chem. Soc.* **1988**, *110*, 6536–6541.
59. (a) Kwok, S. W.; Fotsing, J. R.; Fraser, R. J.; Rodionov, V. O.; Fokin, V. V. *Org. Lett.* **2010**, *12*, 4217–4219; (b) Hu, M.; Li, J.; Yao, S. Q. *Org. Lett.* **2008**, *10*, 5529–5531.
60. Pokhodylo, N. T.; Matiychuk, V. S.; Obushak, M. D. *Synthesis* **2009**, *14*, 2321–2323.
61. Nosková, V.; Džubák, P.; Kuzmina, G.; Ludková, A.; Stehlík, D.; Trojanec, R.; Janošťáková, A.; Kořínková, G.; Mihál, V.; Hajdúch, M. *Neoplasma* **2002**, *49*, 418–425.