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Syntheses and biological activity of bisdaunorubicins

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Abstract—To study the length and flexibility of the linkers between two monomers of bisdaunorubicins for their activity against cancer cells, seven bisdaunorubicins were rationally designed and synthesized through click chemistry. Their cytotoxicity was tested in leukemia cells with MTS assay. The results showed that the compounds with short linkers exhibited higher activity than the compounds with long linkers, while the flexibility of the linker also contributed to their activity. These results indicated that the length and flexibility of the linkers between two monomers in bisdaunorubicins are very critical to maintain their activity against cancer cells.

1. Introduction

Many anticancer compounds in clinical use are DNA intercalating agents, which can bind to DNA.[1] These agents bind to specific DNA sequences, form topoisomerase–DNA complex, and cause double strand DNA breaks.[2,3] Drug affinity to DNA is one of the dominant factors in these interactions.[2–4] Therefore, small molecules that show high DNA binding and intercalation capability are expected to provide better anticancer activity.

The anthracycline antibiotics, daunorubicin (daunomycin, DNR) and doxorubicin (adriamycin), are among the most potent anticancer drugs in cancer chemotherapy.[5] The mechanism of action includes DNA intercalation, topoisomerase inhibition, and production of free radicals to attack DNA.[6] However, the clinical effectiveness of anthracycline is limited by their side effects and development of multidrug resistance (MDR).[7,8] Previous studies have been performed to modify anthracycline glycoside antibiotics for generating analogs with high anticancer activity and low toxicity. However, these efforts have only achieved limited success. For instance, MEN 10755 (a disaccharide anthracycline) has a different DNA binding behavior. The crystal structure of the complex between MEN 10755 and DNA hexamer (CGATCG)[9] showed two different DNA binding sites. In one binding site, the disaccharide resides in the DNA minor groove; in the other binding site, the second sugar protrudes from the DNA helix and is linked to guanine of another DNA molecule through hydrogen bonds. This peculiar behavior suggests that the sugar structure may interact with other cellular targets if appropriate modifications are being made.

To increase the DNA binding affinity of anthracycline, the bisintercalator, which is the dimer of anthracyclines, may provide better opportunity to develop a new series of anticancer analogs. The potential advantages of a bisintercalator over its monomeric counterpart include: (1) DNA binding affinity should be greatly enhanced. The binding constant for the bisintercalator is roughly the square of that for the monomer.[10] (2) The interaction area between the bisintercalator and DNA is increased compared to the monomer. In fact, four types of bisanthracyclines have been reported[10–13] (Fig. 1). BA1 links two monomers of daunorubicin through either C13 or C14 positions and exhibits 1.2- to 7.5-fold higher cytotoxicity than daunorubicin.[10] Doxform links two monomers of doxorubicin through 3′-NH_2 group and shows more activity than doxorubicin.[12] Daunorubicin dimer WP631 even overcomes multidrug resistance in cultured cells.[13] However, most of the dimers through squaric acid amide show less activity than monomer.[11]

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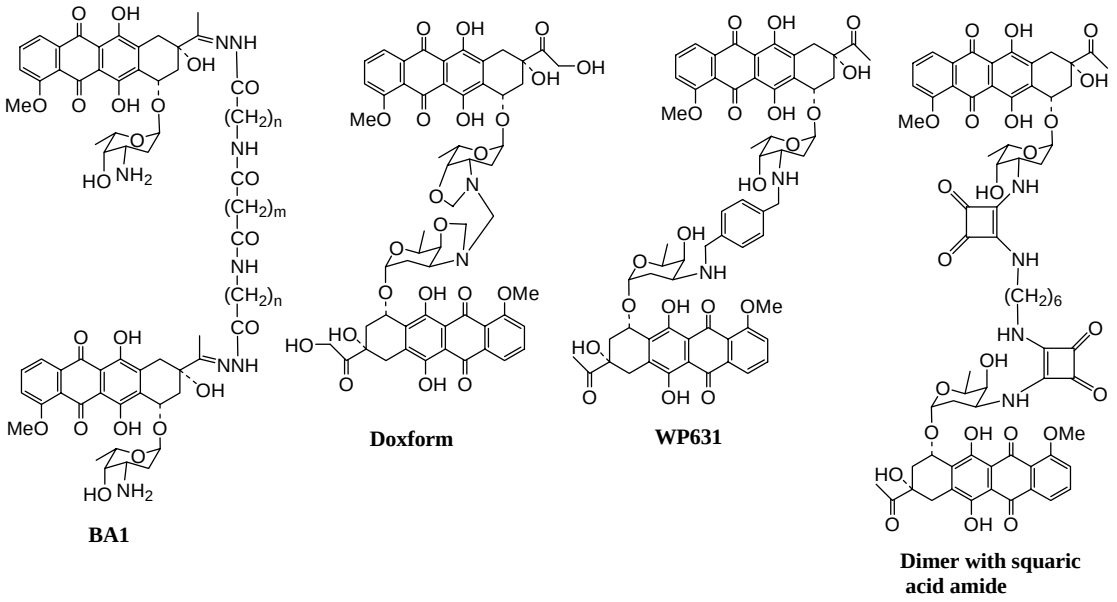


Figure 1. Structures of known bisdaunorubicins.

In addition, the linkers between the two monomers are not well characterized to maintain the anticancer activity of the bisintercalator.

In the present research, we intend to study the length and the flexibility of the linkers between two monomers of bisdaunorubicins for their anticancer activity. The information of anthracyclines binding to DNA provides a foundation for our rational design.[6,14] It was found that the non-covalent complexes of anthracyclines bound to DNA are in a 2:1 stoichiometry of drug to DNA. In such a complex, each drug intercalated at either end of the hexanucleotide, with the daunosamine moieties of the two drug molecules lining the minor groove and pointing toward each other (Fig. 2).[13] The distance between the two sugars is around 6Å. Based on this discovery, we rationally designed to link the two amino groups of daunorubicin to form a bisanthracycline with the potential of bisintercalating into DNA. These bisdaunorubicins were synthesized via click chemistry through the linkers with various lengths and flexibility to test their activities against cancer cells. Huisgen 1,3-dipolar cycloaddition reaction, click chemistry, has proven to be a powerful tool in biomedical research and drug discovery.[15] This reaction has many advantages: (1) the azido group and alkyne are tolerable to most reaction manipulations (no protection and deprotection are necessary); (2) the product can be easily isolated after the reaction. The transformation is especially useful for drug discovery because of its reliability as a linking reaction and the favorable physicochemical properties of triazoles. The triazole products are often associated with biological targets through hydrogen bonding and dipole interactions.[16] In this paper, we report the synthetic method of bisdaunorubicins with various linkers through click chemistry and their biological activity.

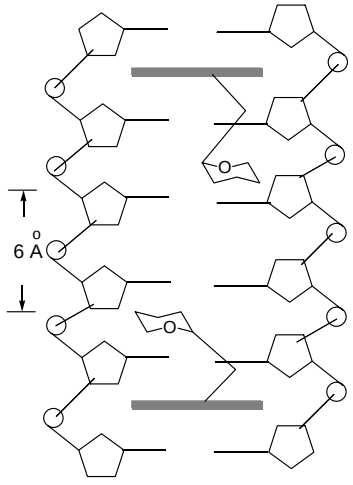


Figure 2. A schematic of the structure of monomeric anthracycline molecules bound to a DNA hexanucleotide as revealed by high-resolution crystallographic structures.

2. Results and discussion

2.1. Chemistry

We designed and synthesized two types of bisdaunorubicins **1**–**7** (Fig. 3) through click reaction. The synthetic routes of compounds **1**–**7** are depicted in Schemes 1 and 2. The synthetic procedures first focused on the intermediates **8**, **9**, **14**, **15**, **17**, and **19**.

Daunorubicin hydrochloride was readily reacted with azido acetic acid in the presence of DCC at rt overnight to produce the intermediate **8** as a red solid in 63% yield. A similar procedure was adopted to give compound **9** in 66% yield. The 3′-azido daunorubicin **15** was prepared from daunorubicin by treatment with TfN_3 solution (made by using 2 equiv of Tf_2O[17]) in 70% yield.

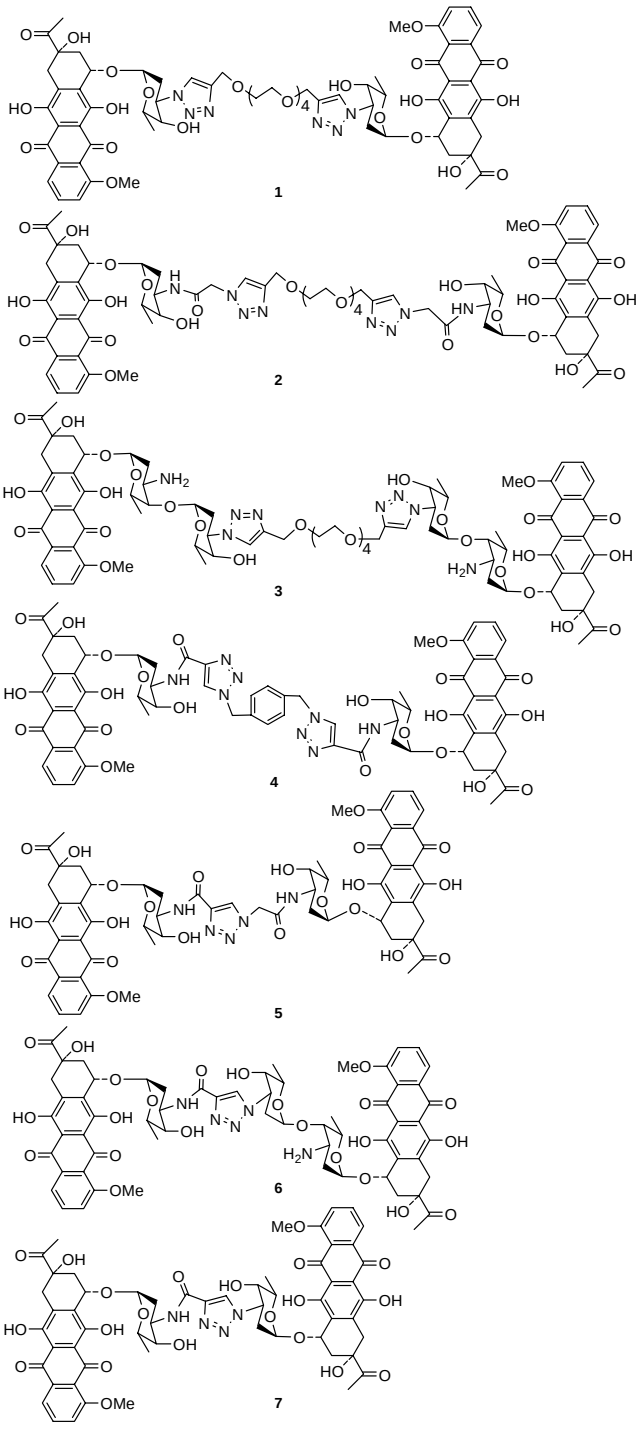


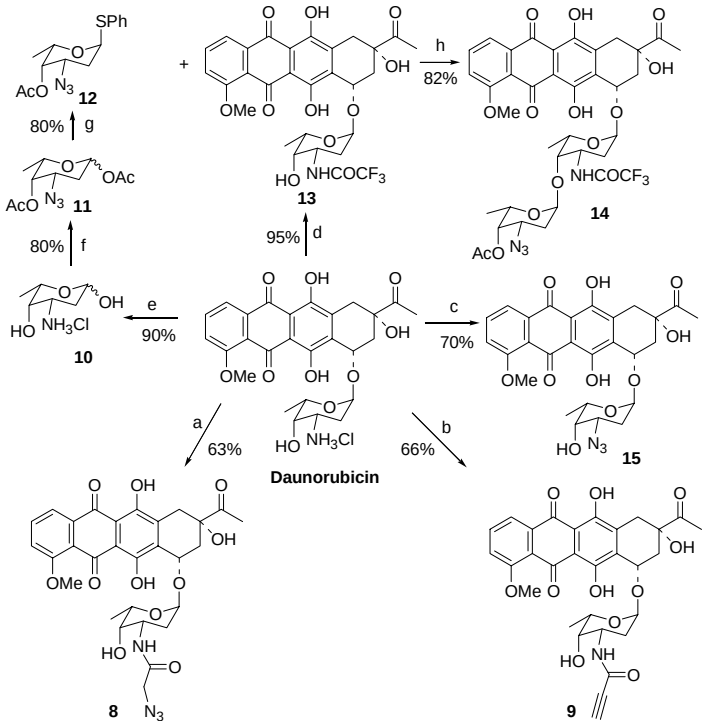
Figure 3. Synthetic bisdaunorubicins (**1**–**7**).

To produce bisdaunorubicins containing disaccharide monomer, we designed a disaccharide daunorubicin **14**. The second sugar was produced from daunosamine and linked to the first sugar through an α-glycosidic bond. The rational design of **14** was based on the following considerations: (1) natural disaccharide anthracycline analogs isolated from *Streptomyces* sp. often contain two or more sugars connected through an α (1 → 4) linkage and invariably possess an amino sugar as the first sugar moiety attached to the aglycone.[5,18] (2) intensive structure–activity relationship (SAR) studies on MEN 10755 reveal that the α-orientation of the second sugar is critical for the topo II poisoning ability.[19,20] To make α-glycosylation with 2,6-dideoxy sugars, we anticipate three synthetic challenges: first, the stability of 2,6-dideoxy sugar donor; second, α-selective formation of the glycosidic bond; and finally the known low reactivity of an *axial*-4-hydroxyl group in the glycosyl acceptors.[21] Thioglycosides are stable to normal protecting manipulation in oligosaccharide synthesis.[22,23] Therefore, thioglycoside **12** was chosen as the glycosyl donor. Recently, Hirama reported a direct and efficient α-selective glycosylation protocol for the kedarcidin sugar and L-mycarose using $AgPF_6$ as a activator of 2-deoxythioglycosides.[24] In our research, we found that $AgPF_6$/TTBP (2,4,6-*tert*-butylpyrimidine) is the better promoter system for the synthesis of **14**. Condensation between the thioglycosides **12** and the glycosyl acceptor **13** in the presence of $AgPF_6$/TTBP produced exclusively α-glycoside **14** with 82% yield. It is worth noting that the present method is an efficient method for the synthesis of disaccharide anthracyclines. This protocol overcame the low reactivity of the *axial*-4′-hydroxy group and produced the desired α-glycosidic bond with good yield.

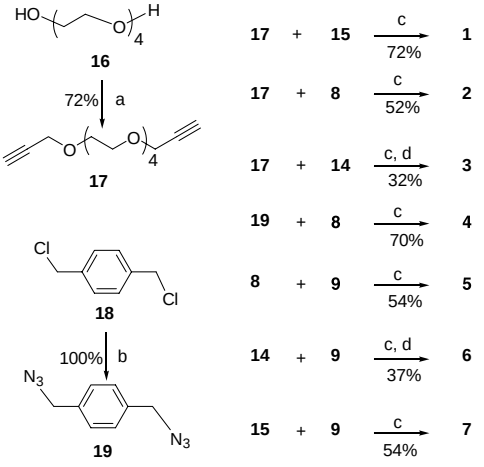
Hydrolysis of daunorubicin with 0.2 M HCl at 90 °C for 1 h gave daunosamine hydrochloride **10** in 90% yield,[25] which was readily converted to the 3-azido sugar **11** in overall yield of 80% according to C.-H. Wong's method.[17] Treatment of **11** with thiophenol in the presence of boron trifluoride diethyl etherate afforded thioglycoside **12** in 80% yield. Trifluoroacetylation of daunorubicin in pyridine gave the glycosyl acceptor **13** in 95% yield.[26] Thus, condensation of **13** with **12** in the presence of $AgPF_6$/TTBP in CH_2Cl_2 at 0 °C for 3 h gave **14** with 82% yield. The spectra of ^{1}H NMR (4.99 ppm, br H-1″) and ^{1}H-^{1}H COSY indicate the newly formed glycosidic bond is α-linkage.

The bisfunctional intermediates **17** and **19** were readily prepared from corresponding tetraethylene glycol **16** and 1,4-bis(chloromethyl)benzene **18**. Treatment of **16** with propargyl bromide in the presence of NaH in THF at 0 °C for 6 h formed **17** with 72% yield. Starting with commercially available **18**, after treatment with sodium azide in the presence of Et_4NBr (4% weight) at 110 °C overnight, compound **19** was obtained in a quantitative yield.

To prepare bisdaunorubicins **1**–**7** through click chemistry, three catalytic systems were explored: CuI/DIPEA (diisopropylethylamine),[27] $CuSO_4{\cdot}5H_2O$/sodium ascorbate,[28] and $(EtO)_3PCuI$/DIPEA.[29] However, only $(EtO)_3PCuI$/DIPEA gave a satisfactory yield. Thus, all the bisdaunorubicins were assembled through click chemistry using this catalytic system in THF at rt. Compounds **1**, **2**, **4**, **5**, and **7** were obtained by a click reaction between the corresponding intermediates with azido group and alkyne in yields of 72, 52, 70, 54, and 54%, respectively. After condensation of **14** with **17** or **9**, the coupling products were deprotected by treatment with 0.1 M NaOH. Thus, compounds **3** or **6** were obtained in the overall yields of 32%, and 37%, respectively.



Scheme 1. Reagents and conditions: (a) $N_3CH_2CO_2H$, DCC, CH_2Cl_2, overnight; (b) $CH{\equiv}CCH_2CO_2H$, DCC, CH_2Cl_2, overnight; (c) K_2CO_3, $CuSO_4$, TfN_3 solution; (d) $(CF_3CO)_2O$/pyridine, −20 °C, 15 min; (e) 0.2 M HCl, 90 °C, 1 h; (f) K_2CO_3, $CuSO_4$, TfN_3 solution; then Ac_2O/pyridine; (g) PhSH, $BF_3{\cdot}Et_2O$, CH_2Cl_2, 0 °C, 2 h; (h) $AgPF_6$, TTBP, CH_2Cl_2, 0 °C, 2 h.



Scheme 2. Reagents and conditions: (a) $CH{\equiv}CCH_2Br$, NaH, THF, 6 h; (b) NaN_3, Et_4NBr (cat), overnight; (c) $(EtO)_3PCuI$, DIPEA, THF, overnight; (d) 0.1 M NaOH, THF, 0 °C.

2.2. Cytotoxicity

The cytotoxicities of these bisdaunorubicins (**1**–**7**) and two intermediates (**8** and **9**) were examined in leukemia cell line K562 cells with MTS assay as described.[30–32] 2000–10,000 cells were incubated with 0.001–5 μM of each compound for 72 h. Then 20 μL MTS/PMS assay solution was added to each well and the absorbance was recorded. The cell survival was calculated as percentage of cell control group without treatment.

Compounds **1**, **2**, and **3** belong to a group with long flexible chains, but compounds **2** and **3** have longer linkers between the two monomers. Compound **1** killed about 35% of K562 cells at the concentration of 25 μM, while compounds **2** and **3** had no cytotoxicity at all at 25 μM concentration (Fig. 4). On the other hand, compounds

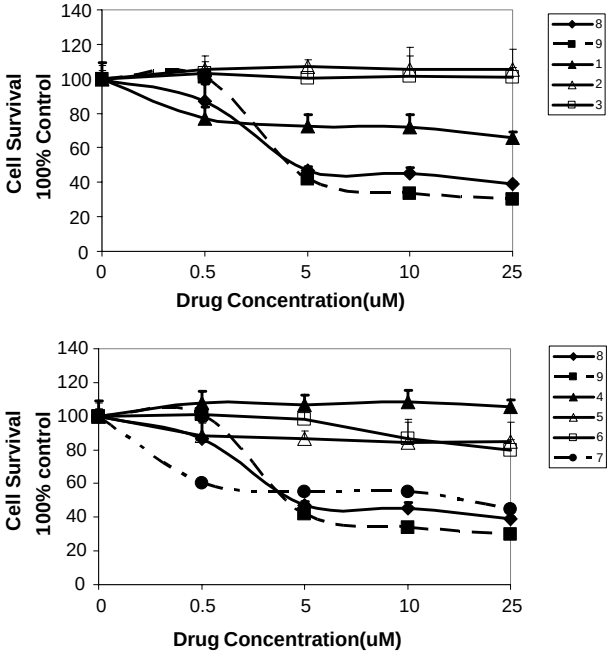


Figure 4. Cytotoxicity of compounds **1**–**9** on leukemia K562 cells by MTS assay (cell survival is compared to control group without treatment of any drugs. The data are average ($X \pm SD$) of six experiments).

4–**7** belong to another group carrying similar relative rigid chains. Compound **7** showed the highest activity with the IC_{50} value of 20 μM, while compounds **4**–**6** (with longer linkers) completely lost their activity even at highest concentration (25 μM) (Fig. 4). These results indicated that the length of the linker between the two monomers in the bisdaunorubicins is very critical for their activity against cancer cells.

Comparing compounds **1**, **4**–**6**, the linker in compound **1** has higher flexibility than those in compounds **4**–**6**. Interestingly, although the linker in compound **1** is even longer than those in compounds **4**–**6**, compound **1** showed significant better activity while compounds **4**–**6** completely lost their activity. This result suggested that the flexibility of the linker may also play a critical role to determine the activity of these bisdaunorubicins. It was also observed that the bisdaunorubicin **7** and monomers (**8** and **9**) exhibited similar activity, but lower than that of the parent compound daunorubicin. This indicated that conversion of the 3′-amino group to an amide is not favored for developing more potent drug candidates. The reservation of the basicity of the amino group is required in the future drug design.

In summary, seven bisdaunorubicins were effectively constructed using the click reactions with $(EtO)_3PCuI$ as catalyst in organic solvent. These bisdaunorubicins have various lengths and flexibility of the linkers between two monomers. The cytotoxicity studies indicated that the compound with shorter linker displays the higher activity against cancer cells, while the flexibility of the linker also contributes to their activity.

3. Experimental section

3.1. Chemistry

3.1.1. General information. All solvents were dried with solvent-purification system from Innovative Technology, Inc. All reagents were obtained from commercial sources and used without further purification. Analytical TLC was carried out on silica gel 60 F_{254} aluminum-backed plates (E. Merck). The 230–400 mesh size of the same absorbent was utilized for all chromatographic purifications. ^1H and ^{13}C NMR spectra were recorded at the indicated field strengths. The high-resolution mass spectra were collected at The Ohio State University Campus Chemical Instrumentation Center.

3.1.2. Procedure for preparation of intermediates 8 and 9. A solution of daunorubicin hydrochloride (1.65 g, 2.93 mmol), azidoacetic acid (0.28 g, 2.77 mmol), and DMAP (0.35 g, 2.86 mmol) in CH_2Cl_2 (100 mL) was stirred at 0 °C for 1 h, and then DCC (0.88 g, 4.3 mmol) was added to the solution. The reaction mixture was then stirred at rt overnight. The sediment was removed and the filtrate was washed with saturated NH_4Cl aqueous solution, water, and dried over Na_2SO_4. After the solvent was evaporated, the crude product was purified on a column of silica gel ($MeOH/CH_2Cl_2$ 1:40) to give compound **8** as a red solid (1.2 g, 63%).

Intermediate **9** was obtained by condensation of daunorubicin hydrochloride with propiolic acid as the same procedure with a yield of 66%.

3.1.3. Procedure for the synthesis of sugar donor 12 from daunorubicin. The daunorubicin hydrochloride (10.0 g) was stirred in 0.2 M hydrochloric acid (800 mL) at 90 °C for 1 h. After cooling, the orange precipitate was collected and dried to give daunorubicinone (6.4 g, 91% yield). The filtrate was evaporated to dryness to give **10** (2.9 g, 90%) as a white solid, which was used in the next reaction without further purification.

Daunosamine hydrochloride **10** (1.84 g, 10 mmol) was dissolved in water (30 mL) and treated with potassium carbonate (2.07 g, 15 mmol) and $CuSO_4{\cdot}5H_2O$ (15 mg, 0.1 mmol). To the mixture were added MeOH (60 mL) and the TfN_3 solution (made by using 2 equiv of Tf_2O[17]). Then, more MeOH was added to homogeneity. The reaction mixture was stirred for 18 h. After the solvent was removed, the residue was acetylated using Ac_2O (30 mL) and pyridine (40 mL) with catalytic DMAP. The solvent was evaporated under vacuum. The residue was dissolved in EtOAc and washed with water. After removal of the solvent, column chromatography of the crude product on a silica gel using EtOAc/hexane (1:4) afforded the product **11** as a yellow oil (2.05 g, 80%).

A solution of **11** (1.9 g, 6.2 mmol), thiophenol (678 mg, 6.2 mmol) in toluene (10 mL) was cooled to 0 °C (ice bath), and then boron trifluoride ethyl etherate (678 μL) was added. The mixture was warmed to rt and stirred for 2 h. After cooling to 0 °C by ice bath, aqueous NaOH (5%, 15 mL) was added, the organic phase was separated, and the aqueous phase was extracted with toluene (50 mL). The combined organic phases were washed with saturated NaCl and evaporated under vacuum to give the crude product. Purification through a column of silica gel (5% Et_2O in hexane) gave the pure compound **12** as a pale yellow oil (80%, α-anomer).

3.1.4. Procedure for the synthesis of sugar acceptor 13 from daunorubicin. Daunorubicin hydrochloride (16.0 g, 2.6 mmol) was stirred in dry pyridine (16 mL) at −20 °C for 0.5 h. Trifluoroacetic anhydride (28 mL) in anhydrous ether (200 mL) was added dropwise over a 1.5 h period. After 0.5 h, water (250 mL) was added and stirring was continued for additional 0.5 h. The reaction mixture was extracted with ethyl acetate and the extracts were washed with water. After drying over Na_2SO_4, the solvent was evaporated, and the residue was heated at reflux with MeOH (50 mL) for 0.5 h. MeOH was distilled off on the rotary evaporator, and the residue was precipitated from chloroform–pentane to afford **13** as a deep red solid (16.8 g, 95%).

3.1.5. Procedure for preparation of intermediate 14. A mixture of **12** (0.35 g, 1.1 mmol), **13** (1.2 g, 1.9 mmol), TTBP (1.46 g, 5.9 mmol), and molecular sieves (4 Å, <5 microns, freshly activated, 1.8 g) in CH_2Cl_2 (15 mL) was stirred at rt for 2 h under N_2 and then

cooled to 0 °C (ice bath). Powdered $AgPF_6$ (1 g, 3.9 mmol) was added and stirred for 3 h. Subsequently, pyridine (7 mL) was added and stirred for a further 0.5 h. Filtration (through a Celite pad), concentration, and chromatographic purification ($MeOH/CH_2Cl_2$ 1:150) provided the products **14** as a red solid (2 g, 82%).

3.1.6. Procedure for preparation of intermediate 15. Daunorubicin hydrochloride (5.24 g, 9.3 mmol) was dissolved in water (30 mL) and treated with potassium carbonate (1.92 g, 13.9 mmol) and $CuSO_4{\cdot}5H_2O$ (14 mg, 88 μmol). MeOH (60 mL) was added to the solution and the TfN_3 solution (made using 2 equiv of Tf_2O[17]). Then, adequate MeOH was added to homogeneity. The reaction mixture was allowed to be stirred overnight. The mixture was diluted with H_2O (100 mL) and extracted with CH_2Cl_2. The combined extractions were dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified through a column of silica gel using $MeOH/CH_2Cl_2$ (1:100–50) to afford **15** as a red solid (70%).

3.1.7. Procedure for preparation of intermediate 17. To a solution of tetraethylene glycol (**16**) (1 g, 5.15 mmol) in THF (30 mL) was added NaH (0.62 g, of 60% suspension in oil, 15.5 mmol) at 0 °C. The resulting mixture was stirred at 0 °C for 0.5 h and then at rt for 0.5 h followed by the addition of propargyl bromide (1.84 g, 15.5 mmol) under N_2 atmosphere. After 6 h, the resultant solution was quenched with water (150 mL) at 0 °C and extracted with EtOAc (500 mL). The organic layer was washed with water and dried over $MgSO_4$. The crude product was purified on a column of silica gel (EtOAc/hexane 1:3) to give **17** as a yellow oil (1 g, 72%).

3.1.8. Procedure for preparation of intermediate 19. The mixture of 1,4-bis(chloromethyl)benzene (5.0 g, 28 mmol), sodium azide (3 equiv), and Et_4NBr (4% weight) was heated to 110 °C and stirred overnight. After cooling, ether (100 mL) was added. The sediment was removed and the filtrate was evaporated to give **19** as a yellow oil in quantitative yield.

3.1.9. Procedure for preparation of bisdaunorubicin 1 and 2. To a solution of **15** (110 mg, 0.20 mmol) and **17** (13.5 mg, 0.05 mmol) in THF (1.5 mL) were added $(EtO)_3PCuI$ (0.2 equiv) and diisopropylethylamine (0.3 equiv). The reaction mixture was stirred at rt for 48 h. Water (2 mL) and $(NH_4)_2S$ solution (22%, 2 mL) were added, and the resultant mixture was stirred for additional 2 h. The product was extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and purification on a column of silica gel (CH_2Cl_2/MeOH 20:1–10:1) provided **1** as a dark red solid (49.7 mg, 72%).

Compound **2** was obtained by condensation of **8** with **17** as the same procedure in a yield of 52%.

3.1.10. Procedure for synthesis of bisdaunorubicin 3. To a solution of **14** (188 mg, 0.22 mmol) and **17** (20 mg, 0.074 mmol) in THF (2 mL) were added $(EtO)_3PCuI$ (0.2 equiv) and diisopropylethylamine (0.3 equiv). The reaction mixture was stirred at rt for 48 h. Water (2 mL) and $(NH_4)_2S$ solution (22%, 2 mL) were added, and the resultant mixture was stirred for additional 2 h. The product was extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and purification on a column of silica gel (CH_2Cl_2/MeOH 30:1) provided a red solid.

A solution of the solid obtained above in THF (5 mL) was cooled in ice bath to 0 °C, and then 0.1 M NaOH aqueous solution (70 mL), which was cooled in advance in ice bath, was added. After stirring for 6–8 h, the reaction mixture was neutralized with 0.1 M citric acid and extracted with $CHCl_3$. The extractions were washed with saturated aqueous $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and chromatographic purification on a column of silica gel (CH_2Cl_2/MeOH 4:1) provided **3** as a dark red solid (45.6 mg, 32% of two steps).

3.1.11. Procedure for synthesis of bisdaunorubicin 4. To a solution of **8** (116 mg, 0.20 mmol) and **19** (10 mg, 0.05 mmol) in THF (2 mL) were added $(EtO)_3PCuI$ (0.2 equiv) and diisopropylethylamine (0.3 equiv). The reaction mixture was stirred at rt for 48 h. Water (2 mL) and $(NH_4)_2S$ solution (22%, 2 mL) were added, and the resultant mixture was stirred for additional 2 h. The product was extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and purification on a column of silica gel (CH_2Cl_2/MeOH 20:1–10:1) provided **4** as a red solid (50 mg, 70%).

3.1.12. Procedure for synthesis of bisdaunorubicin 5 and 7. To a solution of **8** (61 mg, 0.10 mmol) and **9** (58 mg, 0.1 mmol) in THF (2 mL) were added $(EtO)_3PCuI$ (0.2 equiv) and diisopropylethylamine (0.3 equiv). The reaction mixture was stirred at rt for 48 h. Water (2 mL) and $(NH_4)_2S$ solution (22%, 2 mL) were added, and the resultant mixture was stirred for additional 2 h. The product was extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and purification on a column of silica gel (CH_2Cl_2/MeOH 20:1–10:1) provided **5** as a red solid (64 mg, 54%).

Compound **7** was obtained by coupling of **15** with **9** as the same procedure in a yield of 54%.

3.1.13. Procedure for synthesis of bisdaunorubicin 6. To a solution of **14** (82 mg, 0.1 mmol) and **9** (58 mg, 0.1 mmol) in THF (2 mL) were added $(EtO)_3PCuI$ (0.2 equiv) and diisopropylethylamine (0.3 equiv). The reaction mixture was stirred at rt for 48 h. Water (2 mL) and $(NH_4)_2S$ solution (22%, 2 mL) were added, and the resultant mixture was stirred for additional 2 h. The product was extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and

purification on column of silica gel (CH_2Cl_2/MeOH 25:1) provided a red solid.

A solution of the solid obtained above in THF (5 mL) was cooled in ice bath to 0 °C, and then 0.1 M NaOH aqueous solution (70 mL), which was cooled in advance in ice bath, was added. After stirring for 6–8 h, the reaction mixture was neutralized with 0.1 M citric acid and extracted with $CHCl_3$. The extracts were washed with saturated aqueous $NaHCO_3$ solution and dried over Na_2SO_4. Concentration and chromatographic purification on a column of silica gel (CH_2Cl_2/MeOH 10:1) provided **6** (46.7 mg, 37% of two steps).

3.2. Biology

3.2.1. Cell culture. Cell line K562 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and penicillin (100 U/mL) streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The culture media were changed every 2–3 days.

3.2.2. Cytotoxicity of synthesized compounds (MTS assay). K562 leukemia cells (2000–10,000) were seeded in 96-well plates in RPMI-1640 and incubated for 24 h. The exponentially growing cancer cells were incubated with various concentrations of each compound for 72 h at 37 °C (5% CO_2, 95% humidity). After 72 h incubation, tetrazolium[3-(4,5-dimethythiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, 2 mg/mL), and phenazine methosulfate (PMS, 25 μM) were mixed and added directly to the cells. After incubated for 3 h at 37 °C, the absorbance of formazan (the metabolite of MTS by viable cells) was measured at 490 nm.

3.3. Spectroscopic data

3.3.1. *N*-Azidoacetyl daunorubicin (8). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{29}H_{30}N_4O_{11}Na^+$ 633.1803, found 633.1787. ^1H NMR (500 MHz, DMSO-d_6) 13.95 (1H, s, HO-6), 13.20 (1H, s, HO-11), 7.84 (3H, m, H-1, H-2, NHCO), 7.56 (1H, m, H-3), 5.48 (1H, s, OH-8), 5.22 (1H, d, J = 3.3 Hz, H-1′), 4.89 (1H, m, H-7), 4.83 (1H, d, J = 6.1 Hz, OH-4′), 4.19 (1H, q, J = 6.7 Hz, H-5′), 4.00 (1H, m, H-3′), 3.94 (3H, s, MeO-4), 3.77 (1H, d, J = 15.5 Hz, Ha-10), 3.75 (1H, d, J = 15.5 Hz, Hb-10), 3.42 (1H, m, H-4′), 2.89 (2H, s, CH_2N_3), 2.27 (3H, s, H-14), 2.21 (1H, m, Ha-8), 2.06 (1H, m, Hb-8), 1.84 (1H, m, Ha-2′), 1.47 (1H, m, Hb-2′), 1.14 (3H, d, J = 6.5 Hz, H-6′). ^{13}C NMR (125 MHz, DMSO-d_6) 211.7, 186.4, 186.0, 160.5, 155.6, 154.7, 151.9, 134.7, 133.8, 133.7, 120.1, 110.9, 110.6, 100.2, 76.5, 76.0, 73.9, 69.5, 67.9, 66.8, 55.9, 49.2, 48.9, 46.5, 45.7, 34.9, 32.4, 28.7, 24.1, 16.1.

3.3.2. *N*-Propiolyl daunorubicin (9). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{30}H_{29}NO_{11}Na^+$ 602.1633, found 602.1630. ^1H NMR (500 MHz, CD_3OD + $CDCl_3$) 7.79 (1H, d, J = 7.5 Hz, H-1), 7.64 (1H, t, J = 8.0 Hz, H-2), 7.30 (1H, d, J = 8.4 Hz, H-3), 5.37 (1H, d, J = 3.2 Hz, H-1′), 5.06 (1H, br, H-7), 4.08 (2H, m, H-3′, H-5′), 3.94 (3H, s, MeO-4), 3.54 (1H, br, H-4″), 3.29 (1H, s, CCH), 3.28 (1H, d, J = 18.5 Hz, Ha-10), 2.74 (1H, d, J = 18.5 Hz, Hb-10), 2.31 (3H, s, H-14), 2.24 (1H, m, Ha-8), 2.00 (1H, m, Hb-8), 1.87 (1H, m, Ha-2′), 1.68 (1H, m, Hb-2′), 1.21 (3H, d, J = 6.5 Hz, H-6′). ^{13}C NMR (125 MHz, CD_3OD + $CDCl_3$) 212.5, 186.3, 186.2, 166.5, 160.7, 156.1, 154.5, 136.1, 135.5, 134.5, 134.4, 119.9, 119.6, 118.9, 110.6, 110.5, 100.2, 75.1, 70.1, 67.8, 66.6, 56.5, 54.9, 50.5, 45.3, 36.1, 31.5, 29.6, 24.0, 16.9.

3.3.3. Thiophenyl 4-*O*-acetyl-3-azido-L-*lyxo*-hexopyranoside (12). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{14}H_{17}N_3O_3NSa^+$ 330.0883, found 330.0885; ^1H NMR (500 MHz, $CDCl_3$) 7.44 (2H, m, Ar), 7.29 (3H, m, Ar), 5.71 (1H, d, J = 5.6 Hz, H-1), 5.19 (1H, d, J = 2.4 Hz, H-4), 4.45 (1H, q, H-5), 3.18 (1H, m, H-3), 2.44, 2.12 (2H, m, H-2), 2.16 (3H, s, OAc), 1.12 (3H, d, J = 6.3 Hz, H-6).

3.3.4. *N*-(Trifluoroacetyl)daunorubicin (13). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{29}H_{28}F_3NO_{11}Na^+$ 646.1507, found 646.1493. ^1H NMR (500 MHz, $CDCl_3$) 13.98 (1H, s, OH-6), 13.25 (1H, s, OH-11), 8.02 (1H, d, J = 7.6 Hz, H-1), 7.68 (1H, t, J = 8.2 Hz, H-3), 7.37 (1H, d, J = 8.2 Hz, H-3), 6.63 (1H, d, J = 8.4 Hz, $NHCOCF_3$), 5.50 (1H, d, J = 3.9 Hz, H-1′), 5.25 (1H, dd, J = 1.9 Hz, , J = 3.9 Hz, H-7), 4.24 (2H, m, H-3′, H-5′), 4.06 (3H, s, OMe-4), 3.65 (1H, d, J = 2.2 Hz, H-4′), 3.25 (1H, d, J = 18.8 Hz, H-10), 2.92 (1H, d, J = 18.8 Hz, H-10), 2.39 (3H, s, H-14), 2.29 (1H, m, Ha-8), 2.14 (1H, m, Hb-8), 1.94 (1H, m, Ha-2′), 1.80 (1H, m, Hb-2′), 1.28 (3H, d, J = 6.6 Hz, H-6′).

3.3.5. 7-[4-*O*-(4-*O*-Acetyl-3-azido-L-*lyxo*-hexopyranosyl)-2,3,6-trideoxy-3-trifluoroacetoamido-α-L-*lyxo*-hexopyranosyl]-daunorubicinone (14). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{37}H_{39}F_3N_4O_{14}Na^+$ 843.2307, found 843.2331. ^1H NMR (500 MHz, $CDCl_3$) 13.94 (1H, s, HO-6), 13.21 (1H, s, HO-11), 8.60 (1H, d, J = 4.3 Hz, $NHCOCF_3$), 7.99 (1H, d, J = 7.9 Hz, H-1), 7.73 (1H, m, H-2), 7.33 (1H, m, H-3), 5.50 (1H, d, J = 3.4 Hz, H-1′), 5.24 (1H, br, H-7), 5.21 (1H, br, H-4″), 4.99 (1H, br, H-1″), 4.20 (3H, m, H-3′, H-5′, H-5″), 4.04 (3H, s, MeO-4), 3.92 (1H, m, H-3″), 3.58 (1H, br, H-4′), 3.18 (1H, d, J = 18.8 Hz, Ha-10), 2.90 (1H, d, J = 18.8 Hz, Hb-10), 2.38 (3H, s, H-14), 2.17 (1H, m, Ha-8), 2.17 (3H, s, OAc). 2.13 (2H, m, Ha-2″, Hb-8), 2.02 (1H, m, Hb-2″), 1.86 (1H, m, Ha-2′), 1.77 (1H, m, Hb-2′), 1.27 (3H, d, J = 6.4 Hz, H-6′), 1.14 (3 H, d, J = 6.6 Hz, H-6″). ^{13}C NMR (125 MHz, $CDCl_3$) 211.8, 187.1, 186.7, 170.3, 161.0, 156.3, 155.8, 150.4, 135.7, 135.5, 134.3, 133.8, 124.3, 120.9, 119.8, 118.4, 111.5, 111.4, 100.4, 100.2, 80.9, 76.6, 69.9, 69.7, 67.4, 67.3, 56.7, 53.9, 45.6, 35.1, 33.5, 30.8, 30.1, 24.8, 20.7, 17.3, 16.4.

3.3.6. 7-(3-Azido-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)-daunorubicinone (15). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{27}H_{27}N_3O_{10}Na^+$ 576.1589, found 576.1612. ^1H NMR (500 MHz, $CDCl_3$) 13.95 (1H, s, HO-6), 13.19 (1H, s, HO-11), 7.98 (1H, d, J = 7.4 Hz, H-1), 7.74 (1H, t, J = 8.2 Hz, H-2), 7.36 (1H, d, J = 8.4 Hz,

H-3), 5.54 (1H, d, J = 3.6 Hz, H-1′), 5.23 (1H, d, J = 1.9 Hz, H-7), 4.37 (1H, s, HO-9), 4.10 (1H, m, H-5′), 4.05 (3H, s, MeO-4), 3.69 (1H, br, H-4′), 3.60 (1H, m, H-3′), 3.15 (1H, dd, J = 1.6 Hz, J = 18.8 Hz, Ha-10), 2.87 (1H, d, J = 18.8 Hz, Hb-10), 2.38 (3H, s, H-14), 2.28 (1H, m, Ha-8), 2.09 (2H, m, Hb-8, Ha-2′), 1.91 (1H, m, Hb-2′), 1.30 (3H, d, J = 6.6 Hz, H-6′). ^{13}C NMR (125 MHz, $CDCl_3$) 211.5, 186.9, 186.7, 161.1, 156.3, 155.7, 135.7, 135.5, 134.2, 133.9, 120.8, 119.8, 118.5, 111.5, 111.3, 100.6, 76.7, 70.1, 69.5, 67.1, 56.8, 56.7, 34.9, 33.3, 28.5, 24.7, 16.8.

3.3.7. 7,10,13,16-Pentaoxanonadeca-1,18-diyne (17). ^{1}H NMR (250 MHz, $CDCl_3$) 4.21 (4H, s, C≡CCH_2O), 3.60 (16H, m, OCH_2CH_2O), 2.35 (2H, s, C≡ CH).

3.3.8. 1,4-Bis(azidomethyl)benzene (19). ^{1}H NMR (250 MHz, $CDCl_3$) 7.33 (4H, s, Ar-H), 4.34 (4H, s, CH_2).

3.3.9. 1,15-Bis(1-(daunorubicin-3′-yl)-[1,2,3]triazol-4- yl)-2,5,8,11-tetraoxapentadecane (1). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{68}H_{76}N_6O_{25}Na^+$ 1399.4752, found 1399.4684. ^{1}H NMR (500 MHz, $CDCl_3$) 13.89 (2H, s, HO-6), 13.09 (2H, s, HO-11), 7.91 (2H, d, J = 7.6 Hz, H-1), 7.69 (2H, t, J = 8.2 Hz, H-2), 7.62 (2H, s, H-triazole), 7.29 (2H, d, J = 8.5 Hz, H-3), 5.61 (2H, d, J = 3.2 Hz, H-1′), 5.21 (1H, br, H-7), 4.86 (2H, m, H-3′), 4.64 (2H, d, J = 6.1 Hz, HO-4′), 4.50 (2H, d, J = 12.3 Hz, OCH_2-triazole), 4.44 (2H, d, J = 12.3 Hz, OCH_2-triazole), 4.37 (2H, s, HO-9), 4.30 (2H, q, J = 6.5 Hz, H-5′), 4.04 (2H, d, J = 5.6 Hz, H-4′), 3.98 (6H, s, MeO-4), 3.58 (16H, m, –OCH_2CH_2O–), 3.14 (2H, d, J = 18.5 Hz, Ha-10), 2.84 (2H, d, J = 18.5 Hz, Hb-10), 2.51 (2H, m, Ha-8), 2.39 (6H, s, H-14), 2.35 (2H, m, Hb-8), 2.10 (2H, m, Ha-2′), 1.98 (2H, m, Hb-2′), 1.35 (6H, d, H-6′). ^{13}C NMR (125 MHz, $CDCl_3$) 211.9, 186.8, 186.4, 160.9, 156.3, 155.6, 144.0, 135.7, 135.3, 134.4, 133.9, 121.9, 120.7, 119.7, 118.4, 111.3, 111.2, 100.3, 76.6, 70.6, 70.5, 70.0, 69.8, 68.8, 67.7, 64.4, 57.1, 56.6, 35.1, 33.2, 29.2, 24.8, 16.9.

3.3.10. 1,15-Bis(1-(daunorubicin-3′-*N*-yl-carbonylmethyl)-[1,2,3]triazol-4-yl)-2,5,8,11-tetraoxapentadecane (2). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{72}H_{82}N_8O_{27}Na^+$ 1513.5182, found 1513.5233. ^{1}H NMR (400 MHz, DMSO-d_6) 8.14 (2H, d, J = 8.0 Hz, H-1), 7.92 (2H, s, H-triazole), 7.76 (4H, m, H-2, NHCO), 7.49 (2H, m, H-3), 5.45 (2H, s, HO-9), 5.25 (2H, br, H-1′), 5.05 (2H, br, H-7), 4.91 (2H, m, H-3′), 4.87 (2H, d, J = 6.1 Hz, HO-4′), 4.47 (4H, s, OCH_2-triazole), 4.19 (2H, q, J = 6.5 Hz, H-5′), 4.03 (2H, m, H-4′), 3.91 (6H, s, MeO-4), 3.43 (20H, m, –OCH_2CH_2O–, H-10), 2.89 (4H, s, NCH_2CO), 2.32 (8H, m, H-14, Ha-8), 2.21 (2H, m, Hb-8), 1.93 (2H, m, Ha-2′), 1.55 (2H, m, Hb-2′), 1.16 (6H, d, J = 6.4 Hz, H-6′). ^{13}C NMR (100 MHz, DMSO-d_6) 212.1, 212.0, 186.7, 186.6, 165.1, 161.1, 156.6, 154.9, 144.1, 136.4, 135.9, 134.9, 134.8, 125.6, 120.3, 119.9, 119.2, 111.1, 110.9, 100.7, 75.7, 70.1, 70.0, 69.3, 68.3, 67.1, 63.9, 58.9, 52.0, 46.0, 36.7, 36.6, 32.0, 30.1, 24.5, 17.4.

3.3.11. 1,15-Bis(1-(1-(daunorubicin-4′-*O*-yl)-2,3,6-trideoxy-L-*lyxo*-hexopyranos-3-yl)[1,2,3]triazol-4-yl)-2,5,8,11-tetraoxapentadecane (3). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{80}H_{98}N_8O_{29}Na^+$ 1657.6332, found 1657.6334. ^{1}H NMR (400 MHz, DMSO-d_6) 8.07 (2H, s, H-triazole), 7.80 (4H, m, H-1, H-2), 7.55 (2H, d, J = 8.1 Hz, H-3), 5.37 (2H, br, H-1′), 5.23 (2H, br, H-1″), 5.16 (2H, d, J = 4.3 Hz, H-7), 5.10 (4H, m, HO-9, H-3′), 4.85 (2H, br, HO-4″), 4.55 (4H, s, OCH_2-triazole), 4.34 (2H, q, J = 6.6 Hz, H-5′), 4.15 (2H, q, J = 6.5 Hz, H-5″), 3.94 (4H, s, MeO-4), 3.73 (2H, br, H-4′), 3.59 (18H, m, H-4″, OCH_2CH_2O), 3.20 (4H, m, NH_2-3′), 2.97 (2H, m, H-3″), 2.52 (2H, m, Ha-8), 2.27 (6H, s, H-14), 2.14 (2H, m, Hb-8), 1.99 (4H, m, H-2″), 1.71 (2H, m, Ha-2′), 1.15 (2H, m, Hb-2′), 1.17 (6H, d, J = 6.3 Hz, H-6′), 1.09 (6H, d, J = 6.3 Hz, H-6″). ^{13}C NMR (100 MHz, DMSO-d_6) 212.0, 186.5, 186.4, 161.2, 161.1, 156.6, 154.9, 143.9, 136.5, 136.4, 136.0, 135.2, 135.1, 123.2, 123.1, 120.3, 119.3, 111.1, 110.9, 100.7, 98.7, 75.7, 70.3, 70.2, 70.1, 69.5, 69.0, 67.7, 67.4, 64.2, 64.1, 56.9, 56.7, 48.6, 36.4, 29.8, 29.7, 24.5, 17.8, 17.3.

3.3.12. 1,4-Bis-(4-(daunorubicin-3′-*N*-yl-carbonyl)-[1,2,3]triazol-1-yl-methyl)benzene (4). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{68}H_{66}N_8O_{22}Na^+$ 1369.4184, found 1369.4224. ^{1}H NMR (400 MHz, DMSO-d_6) 13.91 (2H, s, HO-6), 13.17 (2H, s, HO-11), 8.54 (2H, s, H-triazole), 7.81 (4H, m, H-1, H-2), 7.65 (2H, d, J = 6.8 Hz, NHCO), 7.55 (2H, d, J = 6.4 Hz, H-3), 7.29 (4H, s, H-Ph), 5.57 (4H, s, CH_2-Ph), 5.46 (2H, s, HO-9), 5.25 (2H, d, J = 1.8 Hz, H-1′), 4.97 (2H, d, J = 4.9 Hz, HO-4′), 4.93 (2H, br, H-7), 4.21 (4H, m, H-3′, H-5′), 3.92 (6H, s, MeO-4), 3.47 (2H, d, J = 3.2 Hz, H-4′), 2.92 (4H, s, H-10), 2.27 (6H, s, H-14), 2.22 (2H, m, Ha-8), 2.12 (2H, m, Hb-8), 1.91 (2H, m, Ha-2′), 1.62 (2H, m, Hb-2′), 1.15 (6H, d, J = 6.1 Hz, H-6′). ^{13}C NMR (100 MHz, DMSO-d_6) 212.1, 186.8, 186.7, 161.2, 159.0, 156.6, 154.9, 143.2, 136.5, 136.1, 136.0, 135.0, 134.9, 128.9, 126.9, 120.4, 120.1, 119.3, 111.1, 111.0, 100.6, 75.7, 70.5, 68.5, 67.2, 56.9, 55.3, 53.2, 45.3, 36.7, 32.1, 30.4, 24.5, 17.3.

3.3.13. 1-(Daunorubicin-3′-*N*-yl-carbonylmethyl)-4-(daunorubicin-3′-*N*-yl-carbonyl)[1,2,3]triazole (5). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{59}H_{59}N_5O_{22}Na^+$ 1212.3544, found 1212.3579. ^{1}H NMR (500 MHz, DMSO-d_6) 13.91 (2H, s), 13.17 (2H, d), 8.36 (1H, s), 8.13 (1H, d, J = 8.1 Hz), 7.81 (4H, m), 7.64 (1H, d, J = 8.6 Hz), 7.54 (2H, m), 5.46 (1H, s), 5.41 (1H, s), 5.23 (2H, br), 5.08 (2H, s), 4.99 (1H, d, J = 6.1 Hz), 4.91 (2H, br), 4.82 (1H, s), 4.17 (3H, m), 3.93 (1H, m), 3.92 (6H, 2s), 3.42 (2H, m), 2.89 (4H, m), 2.26 (3H, s), 2.25 (3H, s), 2.24 (1H, m), 2.08 (2H, m), 1.89 (2H, m), 1.55 (1H, m), 1.45 (1H, m), 1.21 (1H, m), 1.12 (6H, m). ^{13}C NMR (100 MHz, DMSO-d_6) 212.1, 186.8, 186.7, 161.2, 159.0, 156.6, 154.9, 142.7, 136.5, 136.0, 135.1, 134.9, 128.2, 120.5, 120.1, 119.4, 111.2, 111.1, 100.6, 75.7, 75.6, 70.6, 70.4 68.5, 65.5, 57.0, 55.3, 55.3, 52.2, 46.0, 45.3, 36.9, 32.5, 30.9, 30.3, 24.5, 24.4, 17.4.

3.3.14. 1-(1-(Daunorubicin-4′-*O*-yl)-2,3,6-trideoxy-L-*lyxo*-hexopyranos-3-yl)-4-(daunorubicin-3′-*N*-yl-carbonyl) [1,2,3]triazole (6). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{63}H_{67}N_5O_{23}Na^+$ 1284.4119, found 1284.4154. ^{1}H NMR (400 MHz, DMSO-d_6) 8.46 (1H, s), 7.75 (4H,

m), 7.53 (2H, d, $J = 7.6$ Hz), 5.47 (1H, s), 5.35 (1H, s), 5.26 (1H, s), 5.22 (1H, s), 5.13 (4H, m), 4.85 (1H, br), 4.53 (1H, br), 4.29 (3H, m), 4.11 (1H, m), 3.92 (6H, 2s), 3.71 (1H, br), 3.51 (2H, m), 2.95 (5H, m), 2.49 (1H, m), 2.28 (3H, s), 2.24 (3H, s), 1.97 (5H, m), 1.65 (2H, m), 1.51 (1H, m), 1.18 (3H, d, $J = 6.2$ Hz), 1.14 (3H, d, $J = 6.2$ Hz), 1.05 (3H, d, $J = 6.2$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6) 212.2, 212.0, 186.6, 186.54, 186.5, 161.2, 159.3, 156.6, 154.9, 154.7, 142.6, 142.5, 136.5, 136.49, 136.47, 135.9, 135.0, 134.9, 134.85, 134.83, 120.3, 120.2, 119.6, 119.3, 110.9, 110.85, 100.7, 100.6, 98.6, 75.7, 70.6, 68.6, 68.5, 67.1, 57.0, 56.9, 45.2, 36.5, 36.4, 32.1, 32.0, 30.5, 24.5, 24.4, 17.8, 17.4, 17.2.

3.3.15. 1-(Daunorubicin-3′-yl)-4-(daunorubicin-3′-*N*-yl-carbonyl)[1,2,3]triazole (7). HRMS $(M+Na)^+$ (ESI^+) calcd for $C_{57}H_{56}N_4O_{21}Na^+$ 1155.3329, found 1155.3352. ^{1}H NMR (500 MHz, DMSO-d_6) 13.54 (1H, m), 13.45 (1H, m), 12.91 (1H, br), 12.78 (1H, br), 8.51 (1H, s), 7.66 (1H, d, $J = 7.1$ Hz), 7.30 (4H, m), 6.91 (2H, m), 5.40 (1H, s), 5.33 (1H, s), 5.18 (2H, s), 5.10 (1H, d), 5.00 (1H, m), 4.84 (1H, d, $J = 5.5$ Hz), 4.70 (1H, br), 4.36 (1H, d, $J = 6.3$ Hz), 4.19 (2H, d, $J = 6.1$ Hz), 3.78 (1H, d, $J = 4.6$ Hz), 3.55 (1H, br), 3.35 (1H, m), 3.25 (6H, s), 2.80 (4H, m), 2.53 (1H, m), 2.28 (3H, s), 2.25 (3H, s), 2.11 (5H, m), 1.84 (1H, m), 1.58 (1H, m), 1.18 (3H, d, $J = 6.5$ Hz), 1.16 (3H, d, $J = 6.5$ Hz). ^{13}C NMR (100 MHz, DMSO-d_6) 212.0, 211.9, 186.1, 185.5, 185.3, 185.1, 160.4, 159.3, 156.8, 156.1, 154.4, 142.6, 135.8, 135.7, 135.6, 135.1, 134.2, 134.1, 134.0, 125.5, 119.4, 117.9, 117.7, 110.7, 110.6, 110.5, 100.7, 99.6, 75.7, 75.6, 79.3, 70.7, 69.4, 69.3, 68.4, 67.1, 67.0, 57.4, 56.4, 56.3, 45.6, 36.3, 31.9, 31.8, 29.7, 28.9, 24.5, 24.4, 17.5, 17.2.

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