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Pyrrolo[2,1-*c*][1,4]benzodiazepine-β-glucuronide prodrugs with a potential for selective therapy of solid tumors by PMT and ADEPT strategies

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ABSTRACT

Pyrrolo[2,1-*c*][1,4]benzodiazepine-β-glucuronide prodrugs **15a–b**, with a potential for selective therapy of solid tumors by PMT and ADEPT have been designed, synthesized and evaluated for selective cytotoxicity in the presence of the enzyme β-glucuronidase. The prodrugs have been found to possess reduced cytotoxicity compared to the parent moieties, and are excellent substrates for the enzyme, exhibiting cytotoxicity selectively in the presence of the enzyme. Enhanced water solubility and improved stability are the other important outcomes upon modifying these molecules as their prodrugs.

Majority of the anticancer agents are cytotoxic in nature and lack the ability to differentiate between a cancer and normal healthy cell, to kill the cancer cells selectively without causing any adverse effect to the non-target normal tissues.[1] In general, anticancer agents act preferentially on cells that are dividing. Consequently, normal tissues that contain the most rapidly dividing cells are also affected, such as the bone marrow, the gut mucosa and hair follicles.

Enzyme-based therapies like antibody-directed enzyme prodrug therapy (ADEPT),[2] gene directed enzyme prodrug therapy (GDEPT),[3] and prodrug monotherapy (PMT)[4] could improve the selectivity of the anticancer agents by transforming them into non-toxic prodrugs that get selectively activated at the tumor site, leading to localized toxicity. This kind of treatments could allow the use of highly cytotoxic agents with simultaneous sparing of the normal tissues.

Prodrug monotherapy is an approach, wherein an enzyme which is intrinsically present in high concentrations in the malignant tissues, is exploited to selectively activate a comparatively non-toxic prodrug, leading to the formation of a toxic drug. The enzyme β-glucuronidase is reported to be present in high local concentrations in solid tumors compared to the normal tissues.[5] The liberation of lysosomal β-glucuronidase extracellularly leads to the accumulation of the enzyme in the extracellular matrix of the malignant tissues.[6] Targeting the cancer tissues by exploiting the presence of β-glucuronidase in high concentrations is advantageous as the enzyme is not present in blood circulation.[7] The enzyme is confined to lysosomes and therefore is not available for activation of the prodrug in normal tissues. The enzyme is also reported to be present in high concentrations in human breast cancers, which could be helpful for selective therapy of breast cancer.[8]

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs)[9] are a class of small molecules which bind to NH_2 of the guanine in the minor groove of DNA, leading to cytotoxicity. This class of molecules have shown a potential to be developed as anticancer agents, and have also been described to be more suitable for ADEPT strategy compared to mustards, which are the most studied molecules for delivery by this strategy.[10] The lack of selectivity towards cancer tissues is one of the major drawbacks that is associated with anticancer agents, including the PBDs that are in the process of development. The advancement of these molecules as anticancer drugs has been delayed due to various problems relating to high toxicity and reactivity, at different stages of their development.[11] Another shortcoming that makes these compounds less effective in the biological system is their low water solubility leading to decrease in bioavailability.

In an effort to improve the selectivity of PBDs and overall development of these molecules as novel anticancer agents, we have initiated investigations towards the development of PBD prodrugs. In

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this direction two new PBD-β-glucuronide prodrugs **15a–b** (, Fig. 2) with a potential for selective therapy of solid tumors by PMT and ADEPT strategies have been designed synthesized and evaluated for selective toxicity in the presence of the enzyme β-glucuronidase. Compound **15a** is a prodrug of an imine-amide mixed dimer **16a** (Fig. 1) that has been developed in our laboratory and has shown promising anticancer activity,[12] whereas **15b** is a prodrug of benzyl ether analogue of a naturally occurring PBD moiety, DC-81 **16b** (Fig. 1). These prodrugs possess other additional features like enhanced water solubility and stability, apart from their ability to get activated by the enzyme β-glucuronidase.

The preparation of the PBD intermediate for the synthesis of carbinolamine-amide glucuronide prodrug was carried out employing the literature method,[12] by coupling intermediate **2** with excess of 1,5-dibromopentane to obtain **3**, which in turn was coupled with **6**, to yield the intermediate **4**. Compound **4** upon reduction with $SnCl_2$, $2H_2O$ afforded the desired precursor **5a**, which is utilized for the synthesis of the prodrug **15a**. The preparation of DC-81 benzyl ether glucuronide prodrug was carried out according to the literature method.[13]

The synthesis of the β-glucuronide promoiety was carried out starting from glucurolactone (**7**),[14] which was treated with catalytic amount of NaOMe, followed by Ac_2O and $HClO_4$ to obtain the intermediate **8**. This upon treatment with HBr in acetic acid resulted in the formation of compound **9**, which on coupling with 4-hydroxy-3-nitrobenzaldehyde in presence of silver oxide in acetonitrile provided the intermediate **10**. Reduction of **10** with $NaBH_4$, in chloroform and isopropyl alcohol afforded the desired β-glucuronide promoiety **11** (Scheme 1).

The key step of coupling the PBD intermediates **5a–b** with the glucuronide promoiety **11** using triphosgene and dibutyltin dilaurate to yield the carbamates **12a–b**, which upon deprotection of the diethylthioacetal group provided the carbinolamine intermediates **13a–b**. The carbinolamines **13a–b** were deacetylated using NaOMe in methanol at 0–5 °C to provide the intermediates **14a–b**, followed by the hydrolysis of the carboxylic ester afforded the desired PBD β-glucuronide prodrugs **15a–b**[15] (Scheme 2).

The prodrugs were found to possess visibly high water solubility than their corresponding drugs. The enzymatic activation studies showed the prodrugs **15a–b** to be excellent substrates of the

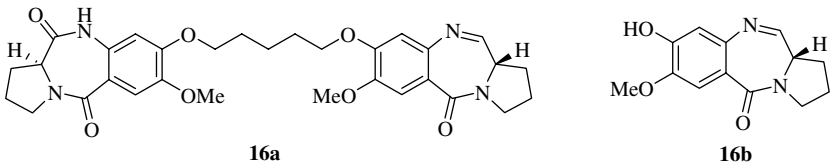


Figure 1. Pyrro[2,1-*c*][1,4]benzodiazepines: **16a** imine-amide mixed dimer; **16b** DC-81.

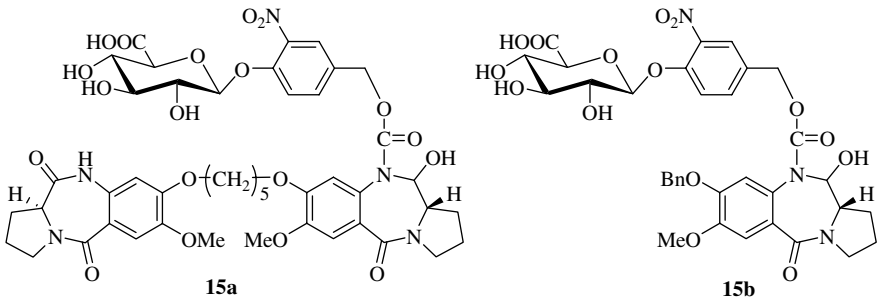
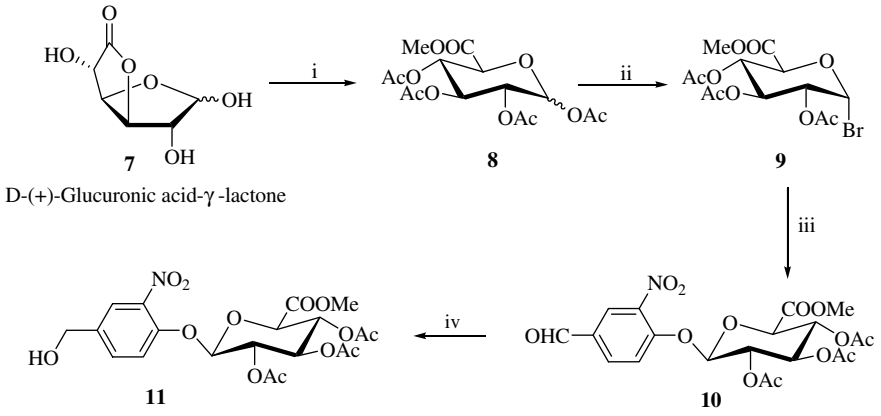
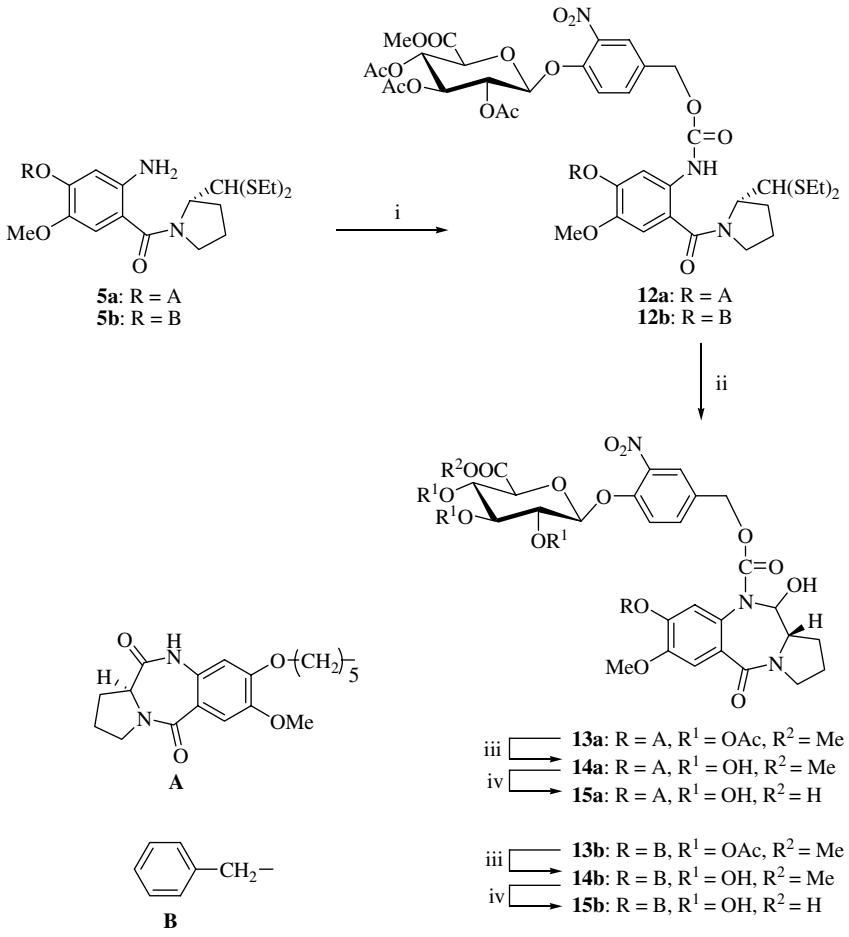


Figure 2. Pyrro[2,1-*c*][1,4]benzodiazepine β-glucuronide prodrugs: **15a** prodrug of imine-amide mixed dimer; **15b** is prodrug of benzyl ether analogue of DC-81.

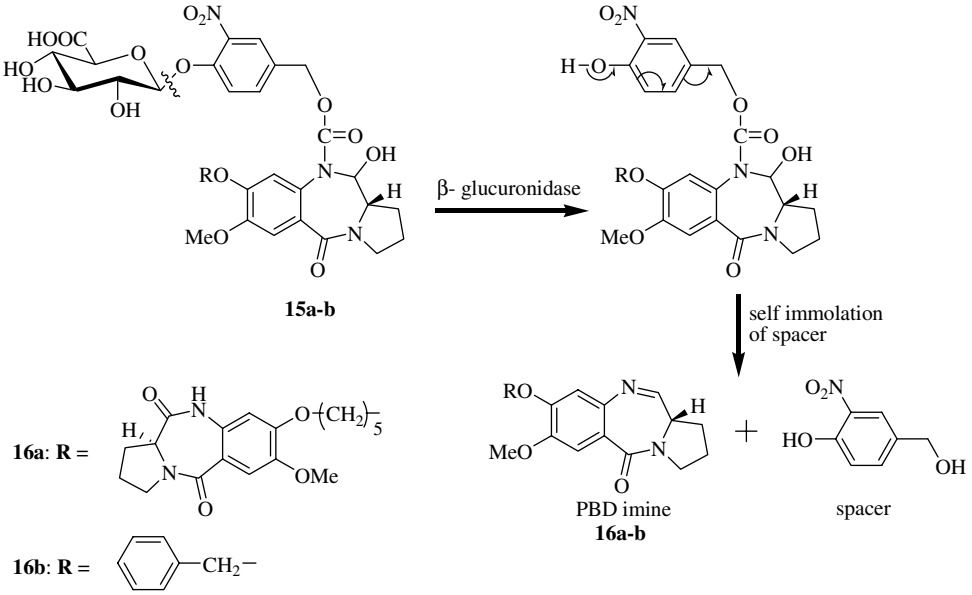


Scheme 1. Reagents and conditions: (i) a—methanol, NaOMe, 60 min, rt, b—Ac_2O, $HClO_4$, rt, overnight, 50%; (ii) 30% HBr/acetic acid, 0–5 °C, overnight, 62%; (iii) Ag_2O, 4-hydroxy-3-nitrobenzaldehyde, acetonitrile, 4 h, 73%; (iv) $NaBH_4$, $CHCl_3$, $(CH_3)_2CHOH$, 0–5 °C, 3 h;78%.



Scheme 2. Reagents and conditions: (i) a—Et_3N, $CO(COCl_3)_2$, 25 min; b—Comp. **11**, cat. dibutyltin dilaurate, **12a**: 74%, **12b**: 71%; (ii) $HgCl_2$, $CaCO_3$, CH_3CN/H_2O, 4:1, **13a**: 77%, **13b**: 84%; (iii) MeOH, MeONa, −15 °C, **14a**: 74%, **14b**: 79%; (iv) LiOH, H_2O/MeOH/THF, 15–20 min, 0–5 °C, **15a**: 76%, **15b: 82%.**

Mechanism of activation



Scheme 3.

enzyme β-glucuronidase. The two β-glucuronide prodrugs were found to be highly stable when incubated at a pH of 7 and 37 °C in a 0.02 M phosphate buffer. However, when these prodrugs were incubated under similar conditions in the presence of *E. coli* β-glucuronidase (250 U), where hydrolyzed completely to form the parent, PBD imine moieties (Scheme 3). The HPLC analysis[16] of the reaction mixture at fixed intervals showed the consumption of the prodrugs and the simultaneous formation of the peaks corresponding to the parent drug as well as 4-(hydroxymethyl)-2-nitrophenol self-immolative spacer. Prodrug **15a** gets activated almost completely in 60 min to form **16a** (Fig. 3), while **15b** gets activated completely in 20 min to form **16b** (Fig. 4).

The pyrrolobenzodiazepine-β-glucuronide prodrugs **15a** and **15b** were evaluated for their selective cytotoxicity using MTT as-

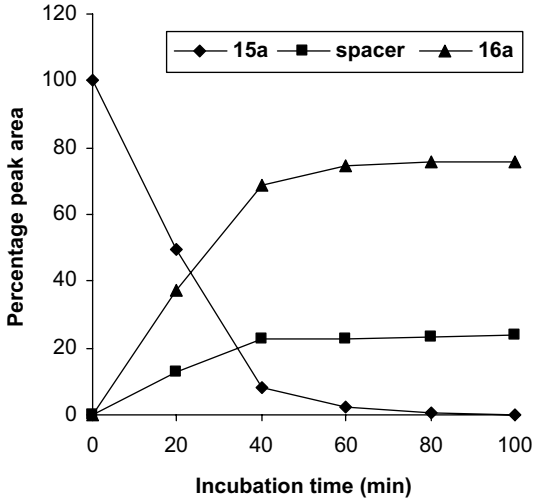


Figure 3. A graph showing the consumption (a decrease in the percentage peak area) of the prodrug (**15a**) with simultaneous formation (an increase in the percentage peak area) of the corresponding active parent PBD moiety (**16a**) and the spacer during the HPLC analysis of activation reaction using β-glucuronidase.

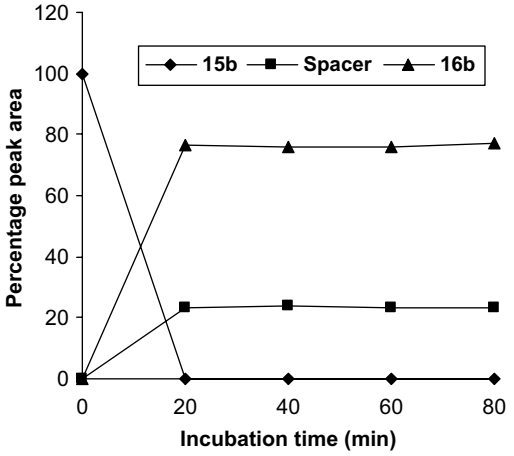


Figure 4. A graph showing the consumption (a decrease in the percentage peak area) of the prodrug (**15b**) with simultaneous formation (an increase in the percentage peak area) of the corresponding active parent PBD moiety (**16b**) and the spacer during the HPLC analysis of activation reaction using β-glucuronidase.

Table 1
Selective cytotoxicity of the prodrugs in HT 29 cell line

Prodrug	IC_{50} (μmol)	IC_{50} (μmol) in the presence of β-glucuronidase	Activation factor (QIC_{50})	IC_{50} (μmol) of the parent PBD
15a	2690	12.88	208.85	8.12
15b	5280	41.68	126.67	30.19

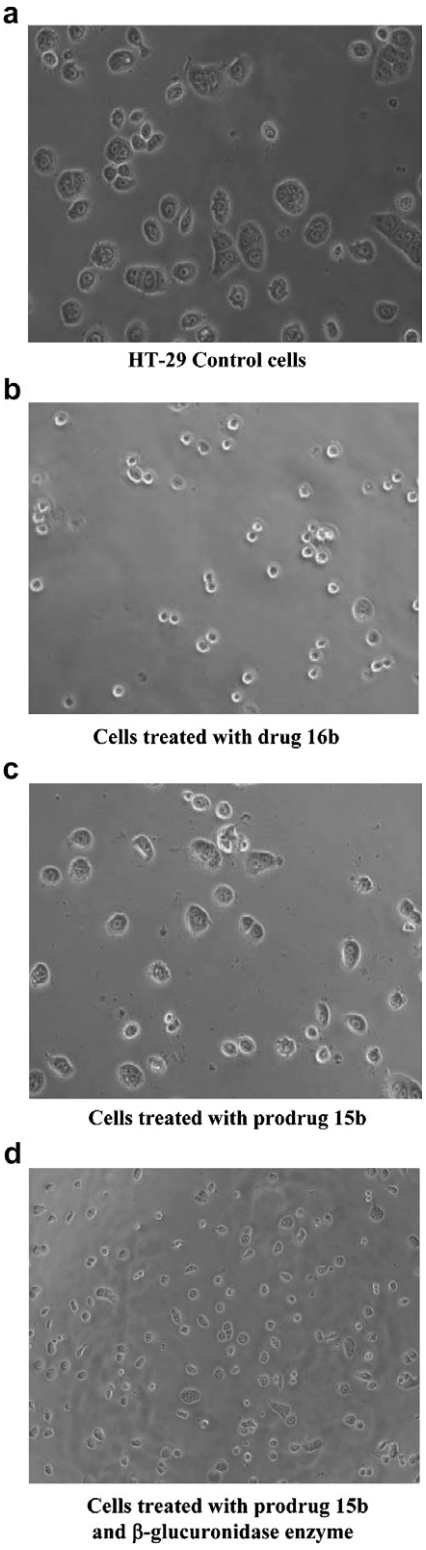


Figure 5. Morphology of HT-29 cells (adenocarcinoma) treated with prodrugs and drugs under microscope after 2 h incubation.

say,[17] in the presence of the enzyme β-glucuronidase using HT 29 colon cancer (adenocarcinoma) cell lines. They were found to possess highly reduced cytotoxicity compared to the parent imines (Table 1). The prodrug **15a** was found to kill 50% of cells (IC_{50}) at a concentration of 2690 μmol, while its parent drug (**16a**) showed an IC_{50} of 8.12 μmol. The prodrug when incubated with the HT 29 cells in the presence of the enzyme β-glucuronidase exhibited an IC_{50} of 12.88 μmol, which is similar to that of the drug. The IC_{50} values of the prodrugs and their corresponding parent moieties shown in Table 1 clearly indicate that the toxicity of the prodrug in the presence of the enzyme is similar to that of the parent drug. The prodrug **15b** also exhibited a reduction in toxicity (IC_{50} = 5280 μmol) when compared to the parent imine (**16b**), which showed an IC_{50} of 30.19 μmol. In the presence of the enzyme **15b** exhibited an IC_{50} value (41.68 μmol) similar to that of the parent moiety.

Both the prodrugs were found to possess highly reduced toxicity and were also good substrates for the enzyme β-glucuronidase. The activity of the prodrugs upon activation by the enzyme approached that of the imines; indicating the complete activation ability of the prodrugs.

The cells after incubating for 2 h with the drug **16b** showed shrinkage (Fig. 5b), on the other hand its prodrug **15b** at the same concentration did not show any shrinkage (Fig. 5c) and the morphology was similar to that of the control cells (Fig. 5a). The prodrug **15b** in the presence of the enzyme β-glucuronidase exhibited cell morphology (shrinkage) similar to that of the cells treated with the drug **16b** (Fig. 5d).

In conclusion, the enzymatic activation and selective cytotoxicity studies of the prodrugs reveal that these molecules possess good activation ability and are potential candidates for use in selective therapy of solid tumors. Compounds **15a** and **15b** possess a potential for use in PMT of solid tumors that over express the enzyme β-glucuronidase.[18] The prodrugs can also be used for antibody-directed enzyme prodrug therapy (ADEPT)[19] of cancer. The other important consequence of these glucuronide prodrugs was highly improved water solubility provided to the drug by the glucuronide functionality.[20]

Acknowledgments

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15. **15a**. mp 163–164 °C; $[\alpha]_D^{25} + 83.33$ (c = 0.3, CH_3OH); ^1H NMR (CD_3OD, 500 MHz): δ 1.32 (t, 2H, J = 7.18 Hz), 1.76–1.91 (m, 4H), 1.96–2.20 (m, 8H), 3.39–3.78 (m, 10H), 3.85 (s, 3H), 3.87 (s, 3H), 3.96–4.17 (m, 4H), 4.80–5.01 (br s, 1H), 5.02–5.11 (m, 1H), 5.26 (d, 1H, J = 12.20 Hz), 5.68 (d, 1H, J = 10.05 Hz), 6.71 (s, 1H), 6.76 (s, 1H), 7.19 (s, 1H), 7.36 (s, 1H), 7.48 (s, 2H), 7.65 (s, 1H), 8.56 (s, 1H, NH); ESI-MS: m/z 964 $[M-H]^-$; HRMS: $[M-H]^-$ calcd for $C_{45}H_{50}N_5O_{19}$ m/z 964.3100, found (ESI) m/z 964.3083; IR (KBr) (νmax cm^{-1}): 3422, 2925, 2855, 1693, 1609, 1526, 1382, 1435, 1061, 1016; Anal. Calcd for: $C_{45}H_{51}N_5O_{19}$: C, 55.96; H, 5.32; N, 7.25%. Found: C, 55.75; H, 5.50; N, 7.03%; **15b**: mp 151–152 °C; $[\alpha]_D^{25} + 6.66$ (c = 0.3, CH_3OH); ^1H NMR (CD_3OD, 500 MHz): δ 1.97–2.19 (m, 4H), 3.38–3.69 (m, 6H), 3.76–3.80 (m, 1H, J = 9.33 Hz), 3.89 (s, 3H), 4.96–5.16 (m, 5H), 5.66 (d, 1H, J = 10.05 Hz), 6.88 (s, 1H), 7.22 (s, 1H), 7.24–7.30 (m, 1H), 7.31–7.36 (m, 2H), 7.37–7.45 (m, 4H), 7.51–7.70 (br s, 1H); ESI-MS: m/z 724 $[M-H]^-$; HRMS: $[M-H]^-$ calcd for $C_{34}H_{34}N_3O_{15}$ m/z 724.1989, found (ESI) m/z 724.2014; IR (KBr) (νmax cm^{-1}): 3424, 2924, 2854, 1673, 1613, 1532, 1460, 1322, 1126, 1021; Anal. Calcd for $C_{34}H_{35}N_3O_{15}$: C, 56.28; H, 4.86; N, 5.79%. Found: C, 56.36; H, 4.62; N, 5.98%.
16. The enzymatic reaction mixtures were analyzed using Phenomenex C18 reverse phase column at 254 nm and 30:70 Acetonitrile–H_2O as eluent system at 1 ml/min flow. The compound **15b** with a retention time of 3.01 min, 4-(hydroxymethyl)-2-nitrophenol spacer 6.35 min and **16b** 22.87 min. **15a** retention time was 3.13 min and **16a** was 22.40 min.
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