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Synthesis and Characterization of Versatile O-glycan Precursors for Cellular O-glycomics

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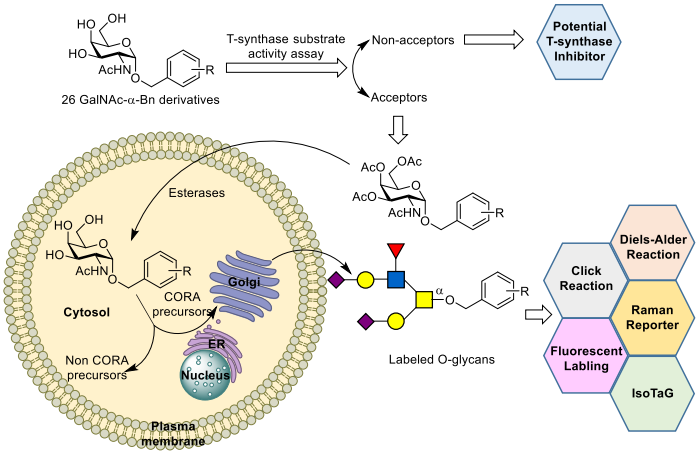
ABSTRACT: Protein O-glycosylation is a universal post-translational modification and plays essential roles in many biological processes. Recently we reported a technology termed Cellular O-Glycome Reporter/Amplification (CORA) to amplify and profile mucin-type O-glycans of living cells growing in the presence of peracetylated Benzyl-α-GalNAc (Ac_3GalNAc-α-Bn). However, the application and development of the CORA method are limited by the properties of the precursor benzyl aglycone, which is relatively inert to further chemical modifications. Here we described a rapid parallel microwave-assisted synthesis of Ac_3GalNAc-α-Bn derivatives to identify versatile precursors for cellular O-glycomics. In total, 26 derivatives, including fluorescent and bioorthogonal reactive ones, were successfully synthesized. The precursors were evaluated for their activity as acceptors for T-synthase and for their ability to function as CORA precursors. Several of the precursors possessing useful functional groups were more efficient than Ac_3GalNAc-α-Bn as T-synthase acceptors and cellular O-Glycome reporters. These precursors will advance the CORA technology for studies of functional O-glycomics.



Keyword: *O-glycosylation, CORA, mucin-type O-glycans, O-glycan precursors, T-synthase*

Protein glycosylation is the most common post-translational modification and plays key roles in diverse fundamental cellular processes[1]. The highly regulated repertoire of cellular glycans are involed in many cellular mechanisms that contribute to health and disease, largely through interactioins with glycan binding proteins and sterically modulating molecular interactions. Due to the inefficiency and the lack of efficient and unbiased strategies for releasing O-glycans from complex samples, current technologies for evaluating O-glycans require relatively large amounts of biological samples for detailed structural analysis, which is a major limitation for functional glycomics. Hence, there is an urgent need to develop a simple and sensitive method to obtain all of the glycans synthesized by cells (the cellular glycome)[2] for investigations in functional O-glycomics.

Mucin type O-glycosylation with GalNAc-α-O-linkage to Ser/Thr/Tyr residues, which occurs on over 80% of proteins[3] that traverse the secretory apparatus, is one of the most common types of protein glycosylation and is important in many normal and pathologic settings[4-7]. In contrast to N-glycans, which can be released enzymatically, O-glycans require chemical strategies for their release, primarily through alkaline β-elimination, which is inefficient and may result in O-glycan degradation. To address these problems, we recently developed a technology termed Cellular O-glycome Reporter/Amplification (CORA)[8], which uses peracetylated Benzyl-α-GalNAc (Ac_3GalNAc-α-Bn) as an O-glycan precursor to amplify and profile the mucin-type O-glycome in living cells, resulting in 100 to 1000-fold increase in sensitivity of detection by mass spectrometry (MS) over traditional methods. Isotope-Cellular O-glycome Reporter Amplification (ICORA) was subsequently developed to quantitatively analyze the O-glycome from cells using stable isotopic labeling[9].

GalNAc-α-Bn at relatively high concentrations (2~5 mM) was first used as an inhibitor of cellular mucin O-glycosylation in human colon cancer cells[10-15], and was also used as an acceptor substrate for *in vitro* T-synthase and Core3GnT activity assay[16-18]. However, we successfully used less than 100 μM concentrations of its peracetylated form, Ac_3GalNAc-α-Bn, as the CORA precursor which was converted by intracellular esterases to Bn-α-GalNAc after being taken up by living cells, and then presumably transported into Golgi lumen as a mucin type O-glycosylation substrate to synthesize Bn-O-glycans with no observed side effect on cellular properties or glycosylation[8]. The precursor Ac_3GalNAc-α-Bn, however, is limited to MS profiling of the O-glycome[8] or to comparing and quantifying O-glycans using ICORA[9]. In order to broaden the application of CORA, we synthesized a variety of differently modified benzyl aglycones to increase the sensitivity of glycan detection by using fluorescent derivatives that permitted us to monitor the purification of the O-glycan derivatives from tissue cultured cells by high performance liquid chromatography (HPLC). In addition, the installation of functional groups to the aglycone provides the potential for covalent coupling the isolated O-glycans to solid surfaces for production of glycan microarrays that present the complete O-glycome of cultured cells for functional O-glycomics.

Herein, we described a rapid parallel synthesis of cellular O-glycan precursors via microwave assisted reaction. By this synthesis method, a library of Ac_3GalNAc-α-Bn derivatives was successfully synthesized, including fluorescent and other useful functional groups. The utility of these precursors was determined by assessing their activity as acceptors for T-synthase and their ability to function as reporter precursors in living cells in culture for profiling O-Glycomes. The new precursors may significantly advance CORA applications, including but not limited to more sensitive analysis, isolation of O-glycans by HPLC with subsequent interrogation of O-glycan libraries by glycan-binding proteins on microarrays, and studies of O-glycosylation pathways by monitoring fluorescence *in vivo*.

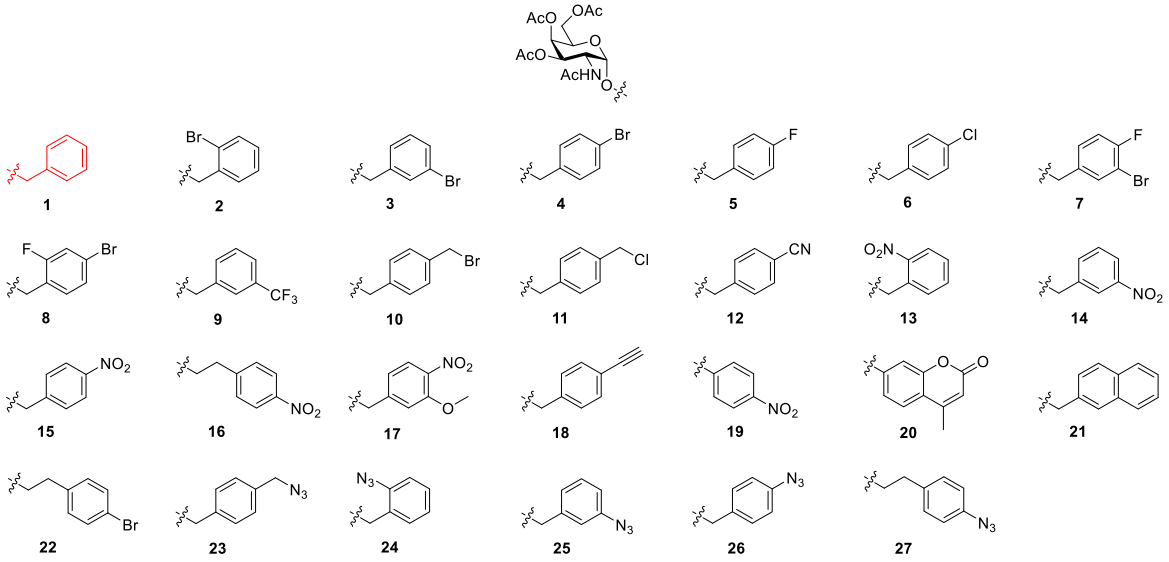
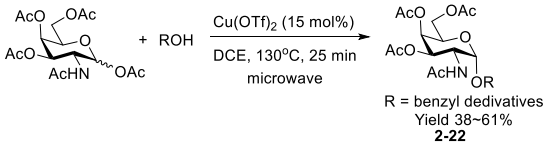


Figure 1. Structure of designed Ac_3GalNAc-α-Bn derivatives.

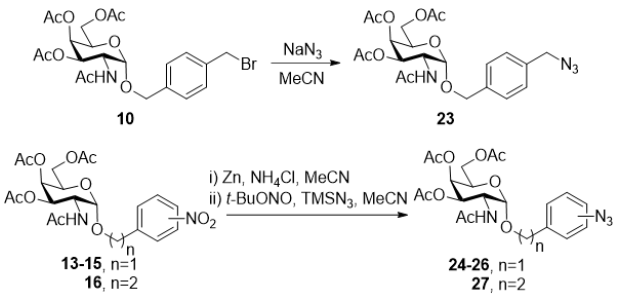
■ RESULTS AND DISCUSSION

Design and Synthesis of CORA precursor candidates. In order to expand the potential applications of CORA, we develop a variety O-glycan precursors that could not only function like Ac_3GalNAc-α-Bn, but also be more versatile, sensitively detected and directly quantified. We designed 26 Ac_3GalNAc-α-Bn derivatives with active functional groups, which are shown in **Figure 1**. The original Ac_3GalNAc-α-Bn was used as the standard for comparison with all of the derivatives with respect to desired properties. These functional groups on the derivatives are designed to be used by well-established methods such as fluorescent labeling strategy (fluorescent group; **20** and **21**)[19], copper-catalyzed azide–alkyne cycloaddition (azide and alkyne groups; **18**, **23**, **24**, **25**, **26** and **27**)[20], Raman reporter strategy (nitrile group; **12**)[21], IsoTaG strategy (bromide; **2**, **3**, **4**, **5**, **7**, **8**, **10** and **22**)[22], and Diels–Alder reaction (alkene group; **18**)[23].

Scheme 1. Microwave assisted synthesis of Ac_3GalNAc-α-Bn derivatives.



Scheme 2. Synthesis of Ac_3GalNAc-α-Bn derivatives with azido group.

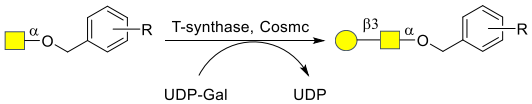


As shown in **scheme 1**, compounds **2-22** were synthesized by microwave reaction described by Beau and co-workers to achieve α-glycosidation from galactosamine pentaacetate[24]. This one-step reaction avoids the use of 2-azido[25], 2,3-trans-oxazolidinone[26], 4,6-O-benzylidenyl[27], 4,6-O-di-tert-butylsilylene[28] or other nonparticipating temporary groups. By controlling the temperature and reaction time, we obtained the desired products in acceptable yields (38~61%). We also tried the reflux method developed by Du and co-workers[29], but this method offered more complex mixture and lower-yield of desired products. Microwave assisted reaction conditions permitted shorter reaction time and better α-stereoselectivity due to higher reaction temperature. Compounds with an azido group cannot be achieved using **scheme 1** directly, using established conditions, because of azide degradation in the presence of $Cu(OTf)_2$. Therefore, compounds **23-27** were prepared as outlined in **scheme 2**. Compound **10** was treated with NaN_3 in MeCN to give compound **23**. The nitro group on compounds **13-16** were modified to azido group to produce compounds **24-27**[30].

Testing of synthesized derivatives as acceptors of T-synthase. We reasoned that in order to produce Bn-O-glycans in the CORA procedure, new precursors would also need to be acceptors of T-synthase, the key enzyme in the pathway for synthesis of most of complex O-glycan structures[31-33]. Furthermore, cell lines without functional *Cosmc*, a molecular chaperone, do not possess a functional T-synthase and cannot secret Bn-O-glycans[8, 31, 34-35]. Therefore, we tested the T-synthase acceptor activity of all compounds by using HPLC to separate and quantify the product of T-synthase.

The acceptor activity was estimated based on the amount of labeled disaccharide generated during the reaction. Each compound shown in **Figure 1** was deacetylated, assayed for acceptor activity, which was reported as activity relative to the control compound Bn-α-GalNAc that was set to a value of 1. The relative T-synthase acceptor activity (RTAA) of compound **2-27** are listed in **Table 1**. The data showed that most of compounds were better acceptors for T-synthase than Bn-α-GalNAc. Compounds bearing a nitro group (**13**, **14**, **15** and **16**) seemed to be better substrates for T-synthase than compounds bearing halogen (**1-12**), alkyne (**18**) or azide groups (**23-27**). The known T-synthase acceptors, pNP-α-GalNAc[12, 18] (compound **19**) and 4-MU-α-GalNAc[19] (compound **20**) were the most active acceptors showing more than 10-fold higher accepter activities compared to Bn-α-GalNAc. Compound **11**, with an RTAA value of 0.59 was considered a weak acceptor, and compound **17**, with an RTAA value of zero had no acceptor activity for T-synthase. Since all of the synthesized compounds possessed an α-GalNAc, there was no obvious reason for any of them not to be an acceptor for T-synthase. Therefore, we investigated the possibility that compound **17** might be an inhibitor of T-synthase. We used the fluorescence-based assay with 4-MU-α-GalNAc as an acceptor of T-synthase[19], and determined that compound **17** was, indeed, an efficient inhibitor of T-synthase *in vitro* with IC_{50} value of 13.4 μM (**Figure S2**).

Table 1. T-synthase acceptor activity of compounds



Compound	RTAA*	Compound	RTAA
1	1		
2	1.81±0.30	**15**	3.01±0.11
3	1.93±0.12	**16**	3.01±0.05
4	1.99±0.10	**17**	0
5	1.55±0.11	**18**	1.64±0.17
6	1.19±0.01	**19**	11.00±0.05
7	1.96±0.07	**20**	13.38±0.45
8	1.67±0.04	**21**	1.91±0.11
9	1.92±0.14	**22**	3.85±0.09
10	2.17±0.34	**23**	1.34±0.01
11	0.59±0.03	**24**	1.59±0.02
12	1.63±0.04	**25**	1.29±0.01
13	4.69±0.20	**26**	1.28±0.01
14	2.26±0.39	**27**	2.15±0.03

*RTAA: relative T-synthase acceptor activity.

Testing of synthesized derivatives in CORA. Due to the promising acceptor activity of most compounds, we tested their CORA precursor activity in living cells. Since the O-glycome of human lung cancer cells has not been reported, we chose the most commonly used non-small lung cancer cell line A549 to test this advanced CORA. We grew A549 cells in the presence of compounds **2-27** at a concentration of 50 μM to see whether they can produce O-glycans corresponding to the known CORA precursor compound **1**[8] (**Figure 2**).

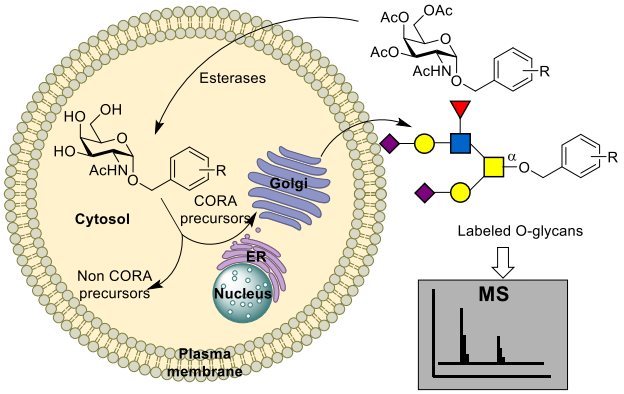


Figure 2. Testing of designed compounds in CORA.

The result showed that 20 of the 26 designed compounds (compounds **2-9**, **13-16**, **18**, **21-27**) functioned as precursors for CORA as demonstrated by their ability to produce O-glycan MS profiles similar to that from compound **1**, where the differences in m/z values were corresponding to the differences in the mass of the different aglycones (**Figure S3** and **Figure 2**). As expected, compound **17**, which was not an acceptor of T-synthase *in vitro* did not function as a CORA precursor *in vivo*, and compound **11**, whose RTAA value was 0.59 also was not converted to O-glycans (**Figure S4**). Interestingly, there were T-synthase acceptor substrates that did not function as precursors for CORA; namely, compounds **10**, **12**, **19** and **20**. Although compound **19** and **20** were the most efficient substrates for T-synthase *in vitro* assays, they generated only sialyl core 1 O-Glycan peaks with low abundance compared with the profile of O-glycans produced using Ac_3GalNAc-α-Bn (**Figure S4**). These data indicate that these compounds either cannot be transported into Golgi lumen, or cannot be processed further *in vivo*. Another T-synthase substrate, compound **12**, merely

produced a few and weak O-Glycan peaks (**Figure S4**). Similar to compound **11,** compound **10** was unable to produce any O-Glycans (**Figure S4**). It is worth noting that the mass of nitro group and azido group containing CORA precursor compounds (compounds **13-16** and **23-27**) were unstable during the permethylation or MALDI-MS-TOF process (**Figure S3c, S3d** and **Figure S5c, S5d**). For example, compound **13**, **15** and **16** produced O-Glycans corresponding to Bn-O-Glycans with a 76 Da mass decrease instead of a 45 Da mass increase (**Figure S3c and S5c**), which suggested that the substituted benzyl group was conversed to methyl group after permethylation reaction due to nitro-derivation and azido-derivation of the Benzyl group. Compounds **25** and **26** produced O-Glycans mainly with a 15 Da mass increase instead of a 41 Da mass increase (**Figure S3d and S5d**), which was presumably caused by the conversion of the azido group (42 Da) to amine group (16 Da).

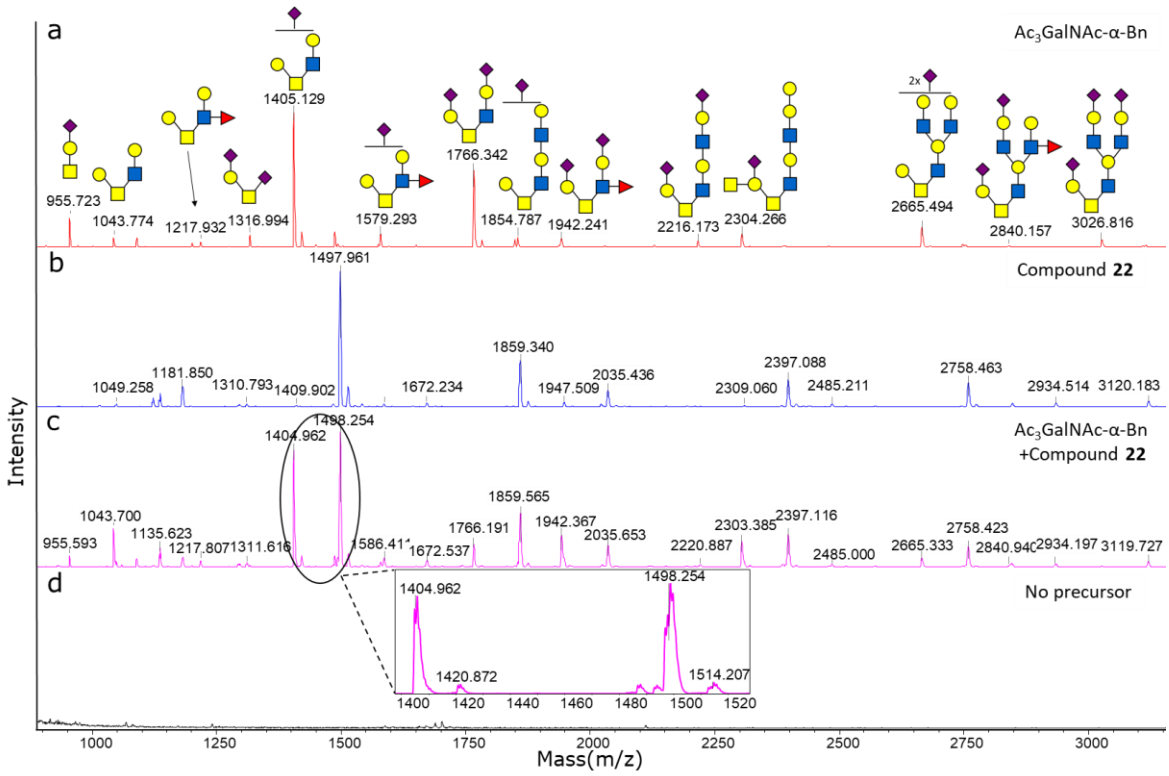
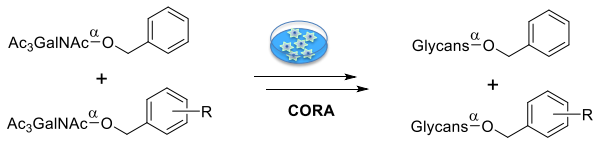


Figure 3. The MS profiles of O-glycans derived from $Ac_3GalNAc$-α-Bn (panel a) and compound **22** (panel b) by A549 cells respectively compared to the MS profile of O-Glycans derived from an equimolar mixture of $Ac_3GalNAc$-α-Bn and compound **22** (panel c) by A549 cells. The ratio of O-glycans MS area provided an estimate of the the relative CORA precursor activity (RCPA).

In order to estimate the relative CORA precursor activity of the compounds tested, we incubated A549 cells with an equimolar concentration of designated compound and $Ac_3GalNAc$-α-Bn at 50 μM for 3 days (**Table 2**). The O-glycans in media were purified, permethylated, and analyzed by MALDI-MS-TOF. A typical result is shown in **Figure 3** where the MS profile of the O-Glycan products generated from a mixture of compound **22** & $Ac_3GalNAc$-α-Bn is shown in panel c. O-glycans derived from compound **22** and O-glycans derived from the $Ac_3GalNAc$-α-Bn were easily distinguished by their mass differences of 93 Da. To estimate CORA precursor activity, the total MS area of O-glycan ions generated form $Ac_3GalNAc$-α-Bn and compound **22** were calculated and the relative CORA precursor activity (RCPA) was defined as the ratio of designated compound O-glycan area/ $Ac_3GalNAc$-α-Bn O-glycan area. In the case, the RCPA of compound **22** was 6.04 and this value together with the calculations for all of the designated compounds is shown in **Table 2**. To correct for differential signal responses in MALDI-MS-TOF for different aglycones, the MS area ratio of the designated compound to $Ac_3GalNAc$-α-Bn was used to normalize the ratios (**Figure S5, Table S2**) so that the RCPA reflects the difference in precursor activities. Every analysis was repeated 3 times. The RCAP data in **Table 2** showed that compound **2-9, 13, 18, 21-27** possessed better CORA precursor activity than standard compound **1** with RCPA values ranging from 1.23 to 6.04. The compounds **14-16** were less efficient than GalNAc-α-Bn, and the RCPA values of compounds **10-12**, **17**, **19**, **20** were zero indicating that they were not effective CORA precursors.

Table 2. CORA precursor activity of compounds



Compound	RCPA*	Compound	RCPA
1	1		
2	3.08±0.57	**15**	0.33±0.04
3	3.09±0.50	**16**	0.17±0.03
4	3.98±0.86	**17**	0

5	2.55±0.46	**18**	1.88±0.07
6	3.44±0.39	**19**	0
7	4.27±0.53	**20**	0
8	2.80±0.35	**21**	1.65±0.79
9	2.92±0.32	**22**	6.04±0.59
10	0	**23**	1.59±0.18
11	0	**24**	1.51±0.71
12	0	**25**	1.89±0.74
13	1.23±0.52	**26**	1.68±0.63
14	0.25±0.10	**27**	2.72±1.97

*RCPA: relative CORA precursor activity.

Structure-Activity Relationships. The RTAA and the RCPA values of all compounds are summarized in **Figure 4**. Compounds **2-9**, which have a halogen substitution on the aromatic ring, all performed stronger as acceptors for T-synthase and as CORA precursors relative to the control compound **1**, which suggested that a halogen on the aromatic ring may contribute to improved interactions with the glycosyltransferase or more efficient uptake into cells or across membranes of the ER and Golgi. Because bromine has a unique isotope pattern in MS, the Br-labeled precursors may have useful applications in identifying the O-glycome similar to IsoTaG glycoproteomics[22].

Derivatives **10** and **11** with bromomethyl and chloromethyl groups, respectively, showed good substrate activity for T-synthase, but don't possess CORA activity; possibly due to substitution reaction of benzyl bromide or chloride with high concentrations of amine and thiol groups on proteins in cells. Precursor **12** possessing a nitrile and compounds **13**, **14**, **15**, **16** and **19** bearing a nitro group on phenyl ring showed better enzymatic acceptor activity than CORA precursor activity, which might be due to these two groups being easy to reduce to amine groups and being consequently metabolized inside the cell[36-37].

Compound **17**, which also possesses a nitro group and an additional methoxy group, was the only tested compound that was not recognized as an acceptor by T-synthase in spite of having a terminal α-linked GalNAc. It is not surprising that this compound did not function as a precursor for CORA. As mentioned above, compound **17** inhibited T-synthase in an *in vitro* assay. However, when we incubated compound **17** at an equimolar concentration with compound **1** with A549 cells in culture, we observed that it had no effect on the MS profile of the GalNAc-α-Bn derived glycans. These data suggest that although compound **17** inhibits T-synthase *in vitro*, it was unable to inhibit the extension of O-glycans on the GalNAc-α-Bn in cells (**Figure.s6**), indicating an inability of compound **17** to cross the Golgi or ER membrane.

Compounds **19** and **20** presented the best acceptor activity for T-synthase in the *in vitro* assay, more than 10-fold greater than the control Bn-α-GalNAc. Compound **19**, PNP-α-GalNAc, and compound **20**, 4-MU-α-GalNAc, are substrates commonly used as substrate for T-synthase enzymatic *in* activity *in vitro* assays[12, 18-19]. It was surprising that these compounds possessed no activity as CORA precursors. Compounds with T-synthase acceptor activity but without CORA precursor activity are presumably not transported into the cell or otherwise unable to access the O-glycan synthesis machinery in the Golgi. Alternatively, they may be metabolized in cytosol.

We were particularly interested in the properties of compounds **18**, **23**, **24**, **25**, **26** and **27**, all of which possess either an alkyne (**18**) or azido (**23-27**) group, which are readily with any functional group tag by click reaction. Compound **21** contains a fluorescent aglycone, which could be an ideal fluorescent labeled precursor without any additional reaction. The fluorescent O-glycans can be directly monitored during HPLC separation permitting the isolation and purification of the individual components of the O-glycans of cultured cells. Fortunately, all of these compounds demonstrated CORA precursor activity that is slightly better than our control precursor Ac_3GalNAc-α-Bn. Compounds **22** and **27** showed better CORA precursor activity and substrate activity than candidates **2-4** and **24-26**, which suggested a longer linker between GalNAc and the aromatic ring is better for glycosyltransferases, especially T-synthase, to access or bind to, and/or accommodate the acceptor.

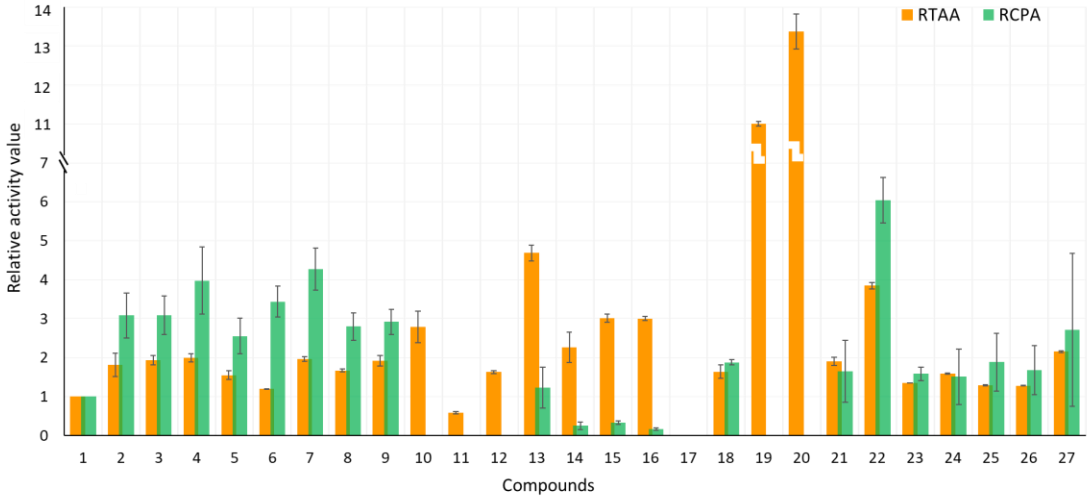


Figure 4. Relative T-synthase acceptor activity (RTAA) and relative CORA precursor activity (RCPA) of all designed precurors.

■ CONCLUSION

In summary, we designed a rapid parallel synthesis of a library of cellular O-glycome precursors via microwave assisted reaction. A total of 26 Ac_3GalNAc-α-Bn derivatives, containing fluorescent moieties and other functional groups, were successfully synthesized by this microwave assisted method. In addition, assays of their activity as acceptors for transfer of galactose to GalNAc *in vitro* by T-synthase and their CORA activity on living cell in culture were followed to evaluate the utility of these derivatives. With these new functional precursors, application of CORA can be expanded to IsoTaG type platforms, direct fluorescence monitoring of O-glycans produced by cells in tissue culture for investigations of O-glycosylation pathways, and post-synthetic derivatizations via click chemistry of the O-glycans of cultured cells at the reducing end of glycans for analysis and isolation of glycans on HPLC. Installation of appropriate functional groups will also permit immobilization of purified O-glycan derivatives as glycan microarrays for subsequent interrogation by glycan-binding proteins. These broad applications will provide useful tools to study functional O-glycomics.

ASSOCIATED CONTENT

Supporting Information. Detailed synthetic procedures and reaction conditions, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry data are available in the supporting information. The description of the assay for T-synthase activity data demonstrating inhibition of T-synthase by compound **17** and the method for normalizing MS intensities to calculate the Relative CORA Precursor Activities are also provided in supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

§QZ and ZL contributed equally to this work. QZ, ZL, XS, TJ, DFS and PGW conceived of the project and designed experiments. QZ and ZL performed experiments. QZ, ZL, TC and VS analyzed the data. QZ and ZL wrote the manuscript. All authors edited the manuscript.

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Notes

The authors declare no competing financial interest.

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