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Novel Synthesis of *N*-Glycosyl Amino Acids Using T3P®: Propylphosphonic Acid Cyclic Anhydride as Coupling Reagent

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Abstract In this paper is presented a novel and simple synthetic pathway for obtaining new protected and unprotected *N*-glucosyl amino acids from 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl amine and Fmoc-L-amino acids. Three methodologies were evaluated, using the coupling reagents: *N,N,N′,N′*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate, diisopropylcarbodiimide and propylphosphonic acid cyclic anhydride. The obtained products using propylphosphonic acid cyclic anhydride showed less undesired species, easy purification and higher yields than the other two methodologies. Deprotection strategies widely used in solid phase peptide synthesis were applied to develop the synthetic pathway reported and achieve the final products. The protected and unprotected *N*-glucosyl amino acids were purified using solid phase extraction chromatography and characterized by high performance liquid Chromatography and nuclear magnetic resonance spectroscopy. Different amino acids (Fmoc-L-Asp(OtBu)OH, Fmoc-L-Phe(OH) and Fmoc-L-Lys(Boc)-OH) have been employed to demonstrate the simple and reproducible coupling methodology using propylphosphonic acid cyclic anhydride. The results showed that new protected and unprotected *N*-glucosyl amino acids can be obtained with high purity and the methodology could be used with any Fmoc-amino acid. The methodology developed could be considered as a synthetic tool for obtaining building blocks for glycopeptide synthesis and potential drugs candidates based on glycoconjugates.

Keywords Glycoconjugate · *N*-glucosyl amino acids · Glycosidic bond · Propylphosphonic acid cyclic anhydride

Introduction

Carbohydrates, one of the most abundant biomolecules in nature, are essential for the sustenance and survival of living things (Varki 1993). These molecules participate in a spectrum of recognition events and binding between surface molecules of pathogens and target cells (Varki 1993). The carbohydrates are involved in cell–cell (cancer), cell–bacteria (*Micobacterium tuberculosis, Escherichia coli, Elicobacter pilory, Vibrio colherae, Bordetelle pertusiis, Pasteurella multocida*, etc) cell–virus (influenza virus, herpes virus, human immunodeficiency virus, ebola virus, etc.), cell-parasite (e.g. *plasmodium falciparum, Leishmania*, etc.), and cell–fungi (*Candida albicans, Cryptococcus neoformans*, etc.) interactions (Osanya et al. 2011; Karlsson 1999; Levitz et al. 2015; Tripathi et al. 2015; Kato and Ishiwa 2015). Carbohydrate and amino acids constitute two important classes of building blocks used by nature to build its vast repertory of molecules (Varki 1993). The sugar amino acids have great potential as multifunctional building blocks in the development of large varieties of designer molecules. The demand for finding new molecules for discovering new drugs is increasing (Chakraborty et al. 2005).

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Successful establishment of infection by bacterial pathogens requires adhesion to host cells, colonization of tissues, and in certain cases cellular invasion (Pizarro-Cerda and Cossart 2006). Conventional therapies involve the use of medicines based on carbohydrates and peptides that produce bacterial death through different mechanisms. Anti-adhesion therapy is an approach focused on preventing the attachment of the bacteria to host tissues, without affecting the bacteria's life (Sharon and Ofek 2001). The adhesion of many pathogenic organisms is mediated by lectins that bind to complementary carbohydrates on the surface of the host tissues (Sharon and Ofek 2001). However, the use of monosaccharides or carbohydrates as possible therapeutic agents show many limitations: requirement of high doses to obtain an effective concentration at the site of action, low residency time, susceptibility to glycosidases, and adverse reactions. Another limitation is that obtaining oligosaccharides implies complex synthetic pathways that affect yields. The development of medicine based on carbohydrates is focused on designing and obtaining small molecules that are easy to synthesize, with high yields and low cost.

In this respect, the amino acid-carbohydrate glycoconjugates can be an interesting approach to develop therapeutic agents that can inhibit bacterial adhesion. The glycosyl amino acids are the smallest glycoconjugates and are the basic building blocks for complex glycoconjugates. The synthesis of *N*-glucosyl amino acids has been focused on obtaining protected derivatives to be used in solid-phase synthesis of glycopeptides (Yao et al. 2010; Van Ameijde et al. 2002; Paolini et al. 2007). However, unprotected *N*-glucosyl amino acids could be considered as a source of new candidates for developing drugs, which has inspired scientists to explore new methodologies for obtaining unprotected *N*-glucosyl amino acids (Monsigny et al. 1998). The synthesis of *N*-glucosyl amino acids involves the formation of the *N*-glycosydic bond between an aminoglucopyranose and the carboxyl group of the amino acid. The glycosidation reaction requires special conditions and the use of toxic reagents that could be difficult to handle. The coupling of amino acids and carbohydrates has been done using many coupling reagents, such as uronium salts (Paolini et al. 2007; Bejugam and Flitsch 2004), carbodiimides (Kornhauser and Keglević 1969; Wang et al. 2010), and other methods (Yao et al. 2010; Van Ameijde et al. 2002; Valentekovic and Keglević 1976; Real-Fernández et al. 2010; Arsequell and Valencia 1997; Kröger et al. 2004). In the present article, we show a new method for coupling amino acids and carbohydrates using the propylphosphonic anhydride (T3P®). This reagent is used in the amide bond formation, and it is very easy to use and combines excellent reaction selectivity and high product purity with good yields. It is an efficient coupling reagent; in the reactions in which it participates, hazardous additives are not required. T3P® can be consider as a green, relatively non-toxic, and non-allergic/non-sensitizing reagent. This is an efficient coupling reagent as well as a water scavenger. It activates the amino acid, forming active ester with the formation of easily removable, non-hazardous, water-miscible propanephosphonic acid as a by-product (Waghmare et al. 2014). The products can be isolated with high yields causing a very limited level of racemization without the use of any additive (Basavaprabhu et al. 2013). The protected and unprotected *N*-glucosyl amino acids were purified by a methdology based on RP-SPE cromatography developed in our laboratory. In the present study, six new *N*-glucosyl amino acids were obtained from D-glucose and three amino acids with different side chain structure, which gives them a different nature in physiological conditions, The products were obtained with high purity and were fully characterized by NMR.

Experimental Methods

General Procedures

Monitoring of Reactions of Compounds ***4, 4b, 4c, 5, 5a, 5b, 5c, 6, 6a, 6b,*** *and* ***6c***

The reactions were monitored by RP-HPLC chromatography using an Agilent Technologies 1260 Infinity Chromatograph. Method 1: Monolithic column Chromolith high-resolution Rp-18e (50×4.6 mm), solvent A: (Water and TFA 0.05%), solvent B: (ACN and TFA 0,05%), gradient elution 15/75/75/15%B in 1/11.5/13.5/17.5 min., Flow: 2 mL/min, wavelength 302 and 210 nm, injection 10 µL (0.1 mg/mL *N*-glucosyl amino acid). Method 2: Eclipse XDB-C18 column (3.5 µm; 4.6×150 mm): solvent A: (water and TFA 0.05%), solvent B: (ACN and TFA 0.05%), gradient elution 15/100/100/15%B in 2/47/54/64 min. Flow: 1 mL/min, wavelength 302 and 210 nm, injection 10 µL (0.1 mg/mL *N*-glucosyl amino acid).

Purification of Compounds ***4, 4c, 5, 5c, 6*** *and* ***6c***

RP-SPE columns were activated in accordance with the supplier's recommendations. The crude product was purified eluting an increasing gradient of ACN. The fractions obtained were analyzed by means of RP-HPLC using method 1.

NMR. The spectra were recorded in $CDCl_3$ and D_2O solutions on an Avance 400 MHz spectrometer.

2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl amine (**3**) was prepared according to standard methods (Mukhopadhyay et al. 2005; Fritz and Schenk 1959; Soli et al. 1999). Obtaining to amine $GlcAc_4$-NH_2 was accomplished via the

corresponding glucosyl azide $GlcAc_4$-N_3 (**2**) as reported by Soli et al. 1999 from 1,2,3,4,6-Penta-*O*-acetyl-α-D-glucopyranose (**1**).

General Procedures for the Coupling Reaction

The *N*-glucosyl amino acids **4, 5**, and **6** were obtained by reaction between 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl amine and Fmoc-L-amino acids, Fmoc-Phe-OH, Fmoc-L-Asp(OtBu)-OH, or Fmoc-L-Lys(Boc)-OH, respectively. The reactions were performed using three different methodologies in order to evaluate the coupling reagents and choose the best one to continue with the reactions. As a model Fmoc-Phe-OH was used, and three methodologies were compared (Fig. 2, *step iv*).

Method (a), (Fig. 2, step iv-a)

Fmoc-Phe-OH (1 mmol) was mixed with TBTU (1 mmol), HOBt (1 mmol) and DIPEA (3 mmol) using DMF as solvent, and then **3** (1 mmol) was added. The mixture was stirred at RT for 16 h. The progress of the reaction was checked by RP-HPLC (Fig. 2a). Then the mixture was dissolved in CH_2Cl_2 and was washed successively with water and saturated $NaHCO_3$. The organic layer was dried with Mg_2SO_4, filtered and concentrated to dryness. Compound **4** was purified via RP-SPE chromatography.

Method (b), (Fig. 2, step iv-b)

Fmoc-Phe-OH (1 mmol) was mixed with **3** (1 mmol) and DIC (1 mmol) using CH_2Cl_2 as solvent. The mixture was stirred at RT for 16 h. The progress of the reaction was checked via RP-HPLC (Fig. 2b). Then the mixture was dissolved in CH_2Cl_2 and was washed successively with water and saturated $NaHCO_3$. The organic layer was dried with Mg_2SO_4, filtered, and concentrated to dryness. Compound **4** was purified via RP-SPE chromatography.

Method (c), (Fig. 2, step iv-c)

Fmoc-Phe-OH (0.65 mmol) was mixed with **3** (1.2 mmol), DIPEA (2 mmol) and EtOAc (10 mL) in a cold bath, and then T3P® 50% EtOAc (1.5 mmol) was added. The mixture was stirred at RT for 16 h. The progress of the reaction was checked via RP-HPLC, and the disappearance of amino acid was observed (Fig. 2b). Then the mixture was dissolved in CH_2Cl_2 and was washed successively with water and saturated $NaHCO_3$. The organic layer was dried with Mg_2SO_4, filtered, and concentrated to dryness. Compound **4** was purified via RP-SPE chromatography.

After analyzing the methodologies, the methodology using T3P® as coupling reagent was chosen for obtaining *N*-glucosyl amino acids, compounds **5** and **6**.

*Fmoc-L-Phe-O-(2,3,4,6-tetra-O-acetyl-β-D-N-glucopyranosyl) (**4**) ($C_{38}H_{40}N_2O_{12}$, MW: 716.22 g/mol)* Compound **4** was obtained as a white solid (195.5 mg, yield 75%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 1.93 (s, 3H, 1 × $COCH_3$), 2.04 (d, J = 9.4 Hz, 6H, 2 × $COCH_3$), 2.11 (s, 3H, 1 × $COCH_3$), 3.12 (s, 2H, Phe β-CH_2), 3.84 (d, J = 7.9 Hz, 1H, H5), 4.13 (d, J = 11.6 Hz, 1H, H-6), 4.22 (t, J = 6.9 Hz, 2H, H-Fmoc), 4.31 (dd, J = 12.8, 3.5 Hz, 1H, H-Fmoc), 4.41 (dd, J = 29.5, 7.4 Hz, 2H, H-Fmoc and Phe α-CH), 4.87 (t, J = 9.5 Hz, 1H, H-2), 5.07 (d, J = 7.5 Hz, 1H, H-4), 5.26 (t, J = 9.2 Hz, 1H, H-1), 5.32 (t, J = 9.5 Hz, 1H, H-3), 6.80 (d, J = 7.4 Hz, 1H, NH), 7.18 (d, J = 5.0 Hz, 1H, Phe-Ar), 7.31 (dt, J = 10.5, 6.0 Hz, 5H, Phe-Ar), 7.43 (t, J = 7.4 Hz, 2H, Fmoc-Ar), 7.59–7.50 (m, 2H, Fmoc-Ar), 7.79 (d, J = 7.5 Hz, 2H, Fmoc-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 20.6 (CH_3), 20.7 (CH_3), 37.3 (CH_2), 47.1 (CH), 56.1 (CH), 61.7 (CH_2), 67.3 (CH_2), 68.1 (CH), 70.5 (CH), 72.6 (CH), 73.6 (CH), 78.2 (CH), 120.0 (CH), 125.0 (CH), 125.1 (CH), 127.1 (CH), 127.2 (CH), 127.8 (CH), 128.8 (CH), 129.5 (CH), 141.3 (C), 143.6 (C), 169.5 (C=O), 169.9 (C=O), 170.4 (C=O), 170.6 (C=O), 171.0 (C=O), 171.4 (C=O).

*Fmoc-L-Asp-(OtBu)-O-(2,3,4,6-tetra-O-acetyl-β-D-N-glucopyranosyl) (**5**) ($C_{37}H_{44}N_2O_{14}$, MW: 740.28 g/mol)* Compound **5** was obtained as a white solid (405.5 mg, yield 57%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 1.46 (s, 9H, 3 × CH_3—OtBu), 2.10 (m, 12H, 4 × $COCH_3$), 2.59 (d, J = 16.8 Hz, 1H, CH_2), 3.09 (d, J = 16.5 Hz, 1H, CH_2, Asp β-CH_2), 3.85 (d, J = 9.9 Hz, 1H, CH, H-5), 4.10 (m, 1H, CH_2, H-6), 4.32 (m, 2H, CH_2, H-6 and Fmoc-CH), 4.52 (m, 2H, Fmoc-CH_2 and Asp α-CH), 4.99 (t, J = 9.4 Hz, 1H, H-2), 5.10 (t, J = 9.7 Hz, 1H, H-4), 5.22 (t, J = 9.2 Hz, 1H, H-1), 5.36 (t, J = 9.5 Hz, 1H, H-3), 7.35–7.28 (m, 2H, H-Ar), 7.44 (t, J = 7.4 Hz, 3H, H-Ar, NH), 7.68 (t, J = 7.7 Hz, 2H, H-Ar), 7.80 (d, J = 7.5 Hz, 2H, H-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 20.5 (CH_3), 20.6 (CH_3), 20.7 (CH_3), 20.8 (CH_3), 28.0 (CH_3), 36.5 (CH_2), 47.0 (CH), 51.3 (CH), 61.6 (CH_2), 67.7 (CH_2), 68.1 (CH), 70.5 (CH), 72.5 (CH), 73.6 (CH), 78.4 (CH), 78.4 (CH), 82.0 (C), 120.1 (CH), 125.2 (CH), 127.2 (CH), 127.8 (CH), 141.3 (C), 143.6 (C), 156.2 (C=O), 169.7 (C=O), 169.9 (C=O), 170.8 (C=O), 171.2 (C=O), 171.4 (C=O), 171.5 (C=O).

*Fmoc-L-Lys-(Boc)-O-(2,3,4,6-tetra-O-acetyl-β-D-N-glucopyranosyl) (**6**) ($C_{40}H_{51}N_3O_{14}$, MW: 797.34 g/mol)* Compound **6** was obtained as a white solid (389.2 mg, yield 92%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 1.27 (s, 4H, 2 × Lys-CH_2), 1.45 (s, 9H, CH_3, 3 × Boc), 1.99 (s, 3H, CH_3, 1 × $COCH_3$), 2.07 (m, 9H, 3 × $COCH_3$), 3.12 (s, 2H,

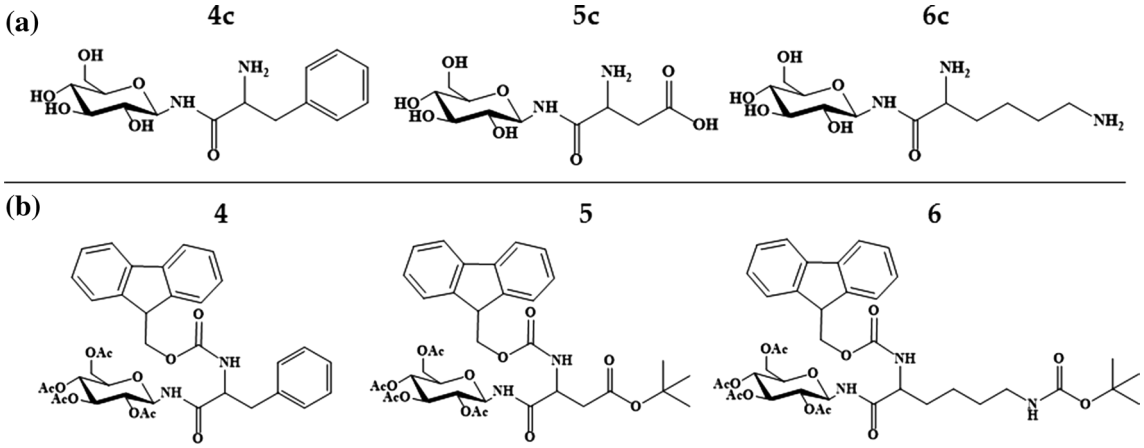

Fig. 1 Design of *N*-glucosyl-amino acids. **a** unprotected target molecules, **b** protected building blocks

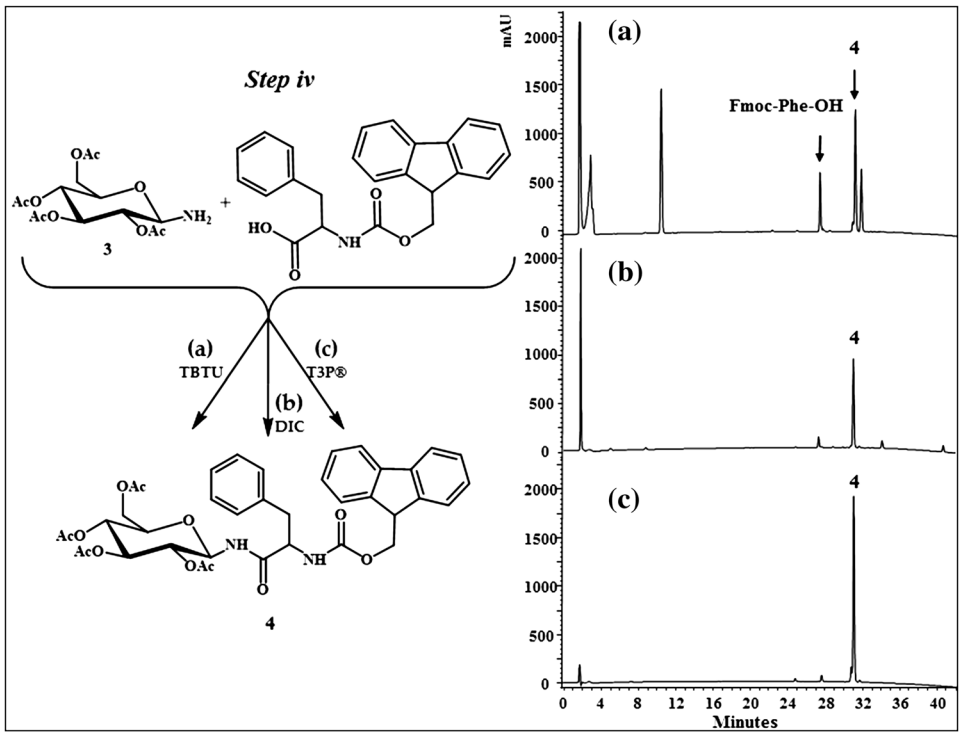

Fig. 2 Comparative analysis via RP-HPLC of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl amine and Fmoc-Phe-OH coupling reaction for obtaining **4** (*step iv*), using three coupling reagents, TBTU (**a**), DIC (**b**) and T3P® (**c**). Reaction time 16 h

CH_2, Lys-CH_2), 3.83 (d, $J=8.2$ Hz, 1H, H-5), 4.12 (m, 3H, CH_2, H-6; Lys α-CH), 4.25 (m, 3H, CH_2, H-6; Fmoc-CH), 4.40 (m, 2H, Fmoc-CH_2), 4.94 (m, 1H, H-2), 5.08 (m, 1H, H-4), 5.24 (m, 2H, H-1 and H-3), 6.99 (d, $J=9.0$ Hz, 1H, NH), 7.34 (t, $J=7.4$ Hz, 2H, Fmoc-Ar), 7.42 (t, $J=7.4$ Hz, 2H, Fmoc-Ar), 7.62 (d, $J=7.2$ Hz, 2H, Fmoc-Ar), 7.78 (d, $J=7.5$ Hz, 2H, Fmoc-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 20.5 (CH_3), 22.3 (CH_2), 28.4 (CH_3), 29.6 (CH_2), 30.9 (CH_2), 34.1 (CH_2), 47.1 (CH), 55.1 (CH), 61.9 (CH_2), 67.2 (CH_2), 68.1 (CH), 70.5 (CH), 73.7 (CH), 78.2 (CH), 90.2 (CH), 120.0 (CH), 125.1 (CH), 127.1 (CH), 127.8 (CH), 141.3 (C), 143.7 (C), 156.2 (C=O), 169.5 (C=O), 169.9 (C=O), 170.1 (C=O), 170.6 (C=O), 170.9 (C=O), 172.3 (C=O).

Methodology for Deprotection of Compounds 4, 5 and 6

To carry out the removal of side chain protective groups OtBu and Boc (Fig. 3, *step v*), compounds **5** and **6** were dissolved in a mixture of TFA/DCM (55% v/v) (Blondelle and Houghten 1993), and the mixture was stirred at room temperature for 2 h. Then the TFA was removed and products **5a** and **6a** were obtained. The removal of the Fmoc group (Fig. 3, *step vi*) was then carried out, dissolving **4,**

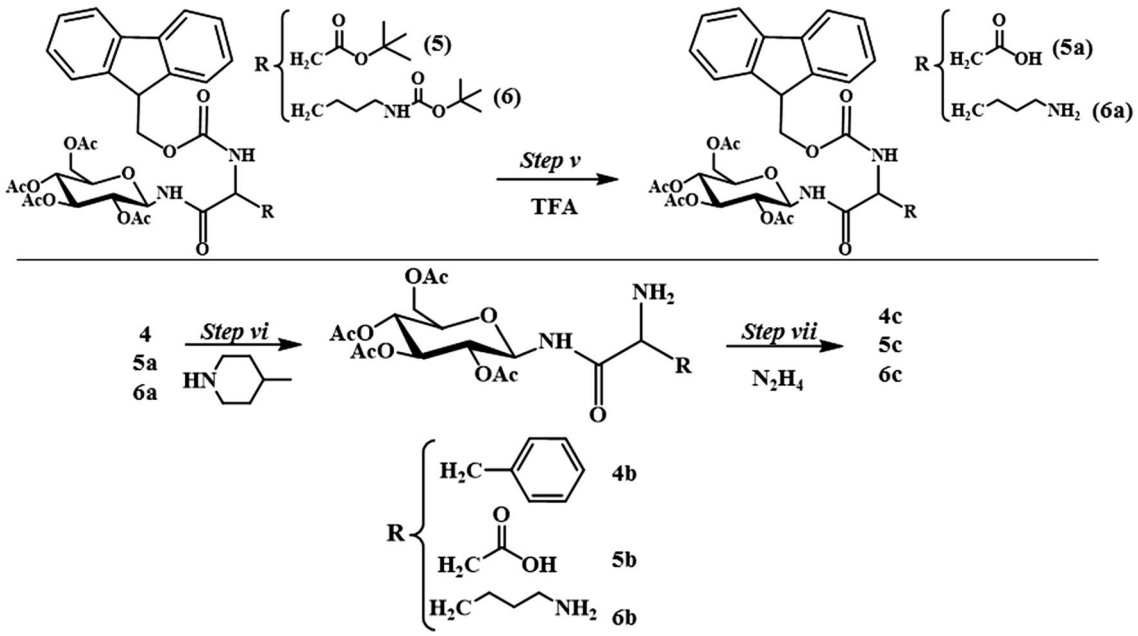

Fig. 3 Synthetic pathway for obtaining unprotected *N*-glucosyl amino acids. The OtBu and Boc groups were removed by treatment with TFA/DCM (*step v*) obtaining **5a** and **6a**. Then **4, 5a**, and **6a** were treated with 4-methyl piperidine and the Fmoc group was removed (*step vi*) for obtaining **4b, 5b** and **6b**, then acetyl groups were removed by treatment with hydrazine hydroxide leads to **4c, 5c** and **6c** (*step vii*), which correspond to unprotected target molecules (Fig. 1a)

5a and **6a** in 4-methylpiperidine/DMF (25% v/v) (Vergel et al. 2014). The mixture was stirred at RT for 3 h and the progress of the reactions was checked via RP-HPLC. Then solvent was removed under reduced pressure and **4b, 5b**, and **6b** were obtained.

Finally, for obtaining **4c, 5c**, and **6c** (target molecules, Fig. 1a), deacetylation of **4b, 5b**, and **6b** was carried out with hydrazine hydroxide, 20%, (Fig. 3, *step vii*) (Khan et al. 1996), the mixture was stirred at RT for 6 h, and the progress of the reactions was checked via RP-HPLC. Then acetone was added and solvent was removed under reduced pressure. The compounds were pre-purified via RP-SPE chromatography, and the products were recrystallized in EtOH.

*L-Phe-O-β-1-N-(D-glucopyranosyl) (**4c**) ($C_{15}H_{22}N_2O_6$, MW: 326.15 g/mol)*

Compound 4c was obtained as a white solid (6.0 mg, yield 32%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 3.06 (m 1H, Phe β-CH_2), 3.20 (m 1H, Phe β-CH_2), 3.33 (m, 2H, 1 × H-2), 3.44 (m, 1H, H-5), 3.66 (m, 1H, CH_2, H-6), 3.82 (m, 1H, CH_2, H-6), 4.16 (m, 1H, Phe α-CH), 4.91 (d, J = 9.2 Hz, 1H, H-1), 7.24 (d, J = 7.8 Hz, 3H, H-Ar Phe), 7.31 (m, 4H, H-Ar Phe).^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 36.9 (CH_2), 54.5 (CH), 60.5 (CH_2), 69.1 (CH), 71.7 (CH), 76.4 (CH), 77.5 (CH), 79.1 (CH), 127.9 (CH), 129.0 (CH), 129.6 (CH), 171.3 (C=O).

*L-Asp-O-β-1-N-(D-glucopyranosyl) (**5c**) ($C_{10}H_{18}N_2O_8$, MW: 294.11 g/mol)*

Compound 5c was obtained as a white solid (5.6 mg, yield 20%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 2.79 (dd, J = 17.7 and 8.3 Hz, 1H, Asp β-CH_2), 2.91 (dd, J = 17.7 and 4.6 Hz, 1H, Asp β-CH_2), 3.33 (t, J = 8.9 Hz, 2H, H-5), 3.49–3.40 (m, 2H, H-2), 3.62 (dd, J = 12.4, 5.2 Hz, 1H, CH_2, H-6), 3.78 (d, J = 13.9 Hz, 1H, CH_2, H-6), 4.25 (dd, J = 8.2, 4.7 Hz, 1H, Asp α-CH), 4.93 (d, J = 9.1 Hz, 1H, 1-H),. ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 35.7 (CH_2), 50.2 (CH), 60.4 (CH_2), 69.1 (CH), 71.7 (CH), 76.3 (CH), 77.6 (CH), 79.3 (CH), 170.1 (C=O), 174.3 (C=O).

*L-Lys-O-β-1-N-(D-glucopyranosyl) (**6c**) ($C_{12}H_{25}N_3O_6$, MW: 307.17 g/mol)*

Compound 6c was obtained as a white solid (42.9 mg, yield 73%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 1.42 (m, 3H, Lys-CH_2), 1.63 (m, 3H, Lys-CH_2), 1.89 (m, 3H, Lys-CH_2), 2.92 (m, 2H, Lys β-CH), 3.34 (m 2H, H-2), 3.52–3.40 (m, 2H, H-5), 3.61 (m, 1H, H.6), 3.79 (d, J = 14.3 Hz, 1H, H-6), 3.99 (t, J = 6.4 Hz, 1H, Lys α-CH), 4.94 (d, J = 9.2 Hz, 1H, 1-H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 21.09 (CH_2), 26.3 (CH_2), 30.1 (CH_2), 39.0 (CH_2), 53.2 (CH), 60.5 (CH_2), 71.8 (CH), 76.4 (CH), 77.8 (CH), 79.4 (CH), 170.9 (C=O).

Results and Discussion

New unprotected *N*-glucosyl amino acids were designed (Fig. 1a). Please note that the selected sugar motif is glucose and the amino acid motif is phenylalanine, aspartic residue, or lysine, whose lateral chain is non-polar aromatic, acidic, and basic, respectively. For all the designed molecules, the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl amine is attached to the amino acid by the α-carboxylic acid group. For obtaining these molecules, it is necessary to use orthogonal protection groups. Thus, the acetyl group was selected for the glucose residue and amino acids were used with the Fmoc/tBu system of protection (Fig. 1b).

The coupling of Fmoc amino acids and glucopyranosylamine $GlcAc_4$-NH_2 was performed using different coupling reagents, specifically (i) TBTU, (ii) DIC, and (iii) T3P®, as will be discussed below (Fig. 2, step iv). Then T3P® was chosen as coupling reagent for obtaining **4, 5** and **6**. In the last step, the protective groups of *N*-glucosyl amino acids were removed by treatment with TFA, 4-methylpiperidine and hydrazine hydroxide (Fig. 3).

Three different methodologies were analyzed and evaluated for obtaining *N*-glucolsyl amino acids, using three coupling reagents *N,N,N′,N′*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) (Fig. 2a), diisopropylcarbodiimide (DIC) (Fig. 2b), and propylphosphoninc anhydride (T3P®) (Fig. 2c). The reactions were performed using Fmoc-Phe-OH as starting reagent and monitored using RP-HPLC, and the results are shown in Fig. 3.

The RP-HPLC analysis (C18 packed column) of compound **4** formation using three different coupling reagents is shown in Fig. 2. The chromatographic profile of the reaction performed using TBTU (Fig. 2a) shows the formation of several species that affect the purification of **4**. The incomplete reaction (at 16 h) can also be seen. The profile for the coupling reaction using DIC (Fig. 2b) shows the formation of two species with a higher retention time, which can produce difficulties in the purification process, besides which the formation of *N*-acylurea precipitate, which causes difficulties for its physical separation and consequently product purification. The profile for the reaction using T3P® shows the disappearance of the amino acid peak and the formation of only one species (Fig. 2c). Table 1 shows obtained yields for Fmoc-L-Phe-OH coupling to $GlcAc_4$-NH_2 using TBTU/HOBt, DIC or T3P®/DIPEA generating **4**. It was observed that T3P® reagent led to higher yields. The reaction using T3P® by-products are water soluble products, which reduces the time of purification. Furthermore, DIC is a toxic reagent, and HOBt has been classified as an explosive compound (Wehrstedt et al. 2005), while T3P® is non-toxic and non-allergenic and can be classified as a green reagent (Waghmare et al. 2014).

Table 1 Coupling of $GlcAc_4$-NH_2 to phenylalanine to obtain product 4

Coupling reagent	Yield (%)
TBTU/HOBt	39
DIC	42
T3P®/DIPEA	75

Reagents: Fmoc-L-Phe-OH (1 mmol), $GlcAc_4$-NH_2 (1 mmol), coupling reagent (1 mmol), DIPEA (3 mmol), 16 h, RT

After obtaining protected *N*-glucosyl amino acids **4, 5**, and **6** using T3P® reagent, the RP-HPLC analysis was done using two chromatographic methods: (1) using a monolithic column Chromolith high resolution RP -18e (new method) and (2) using a packed column XDB—C18 (conventional method). A reduction of retention time, required solvent volume, and analysis time required (run time) was observed. These data show the benefits that a monolithic column can give us in the analysis of hydrophobic molecules, reducing several aspects such as analysis time (60%) and consumption of solvents (50%). The purification method via RP-SPE was simple, fast, and require less solvent volumes, additionally the purified products were obtained with higher yields and similar purity than conventional Flash chromatography. The analysis monitoring via RP-HPLC (using a monolithic column), and some steps of the synthetic pathway fulfill some of the principles of green chemistry.

In general, the synthesis involves less hazardous chemical synthesis and catalysts in the majority of the reactions. It also was designed for energy efficiency. All reactions were done under room temperature and atmospheric pressure conditions. Characterization and purification methods by reverse phase were done considering the use of safer solvents and auxiliaries, which reduces the consumption of organic reagents that can affect human health and the environment.

Our results showed that it was possible obtain the intermediates **4, 5** and **6** with high purity and good yields (50–95%). The results suggesting that the coupling reaction between Fmoc-amino acids and $GlcAc_4$-NH_2 using T3P is affected by the side chain amino acid nature, specifically the reaction with Fmoc-Phe-OH proceeds with 75% of yield, while using Fmoc-Leu-OH the yield was lower (56%) (data no shown). The pathway described here can be applied to the synthesis of protected *N*-glucosyl amino acids, which are the building blocks for *N*-glycopeptide synthesis.

It was also possible to obtain **4c, 5c**, and **6c** with high purity. It was shown that deprotection reactions are the critical point for obtaining unprotected *N*-glucosyl amino acids. The pathway implies sequential deprotection steps

(Fig. 3). First, the side chain protective groups were removed by treatment of *N*-glucosyl amino acids **5** and **6** with TFA/DCM solution. The progress of the reactions was monitored via RP-HPLC; the retention time of **5a** and **6a** was less than **5** and **6**, respectively; this is due to the deprotection reaction, which yields fewer hydrophobic products. When peaks for **5** or **6** disappeared, the reaction was considered finished. The Fmoc group removal was achieved by treatment of **4, 5a**, and **6a** with 4-methylpiperidine in DMF for obtaining **4b, 5b**, and **6b**, respectively. The reactions were monitored by RP-HPLC; the peak of the starting reagent disappeared, while the peak corresponding to the by-product dibenzofulvene adduct was observed. The last step involved the removal of acetyl groups by treatment of **4b, 5b**, and **6b** with Hydrazine hydroxide; this yields **4c, 5c**, and **6c**, respectively. Then the crude products were pre-purified and recrystallized in EtOH. The yields and purity grade suggest that deprotection reactions are affected because of their amino acid side chain nature.

As the spectral data indicate, it was possible to assign all shifts of the hydrogen and carbon atoms of the protected and unprotected *N*-glucosyl amino acids. The glucopyranose protons and carbon atoms of compounds **4, 5**, and **6** exhibit similar shifts; however, the glycosidic bond protons showed different shifts, as well as the protons and carbon atoms of side chains of amino acids. The same behavior was observed in the NMR spectra of compounds **4c, 5c**, and **6c**.

Conclusions

A new synthetic route for obtaining unprotected *N*-glucosyl amino acid was developed using T3P® as coupling reagent for the formation of the amide bond between the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl amine and the carboxy group of Fmoc-L-amino acids. The removal of protective groups of side chains (OtBu and BOC), amino group (Fmoc), and hydroxyl groups (OAc) was achieved. Our interest was to analyze diverse coupling reactions to be used in the synthesis of unprotected *N*-glucosyl amino acids and to demonstrate that this method is suitable for amino acids with different natures. The best results were obtained using propylphosphonic acid T3P®. Dramatic improvements in the consumption of reagents and time of analysis using RP-HPLC and RP-SPE for purification were observed. In this way, some principles of green chemistry can be achieved in the synthetic pathway and the purification methods developed in this paper.

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Compliance with Ethical Standards

Conflict of interest The authors declares that they have no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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