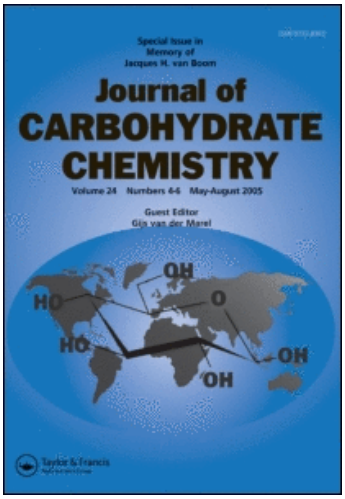

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SYNTHESIS AND IMMUNOSTIMULATING ACTIVITY OF A THIOGLYCOLIPOPEPTIDE GLYCOMIMETIC AS A POTENTIAL ANTI-CANCER VACCINE DERIVED FROM TN ANTIGEN[1]

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ABSTRACT

The Tn epitope is one of the tumor associated *O*-linked cell surface glycopeptides. It is expressed in over 70% of human epithelial cancers such as lung, colon, stomach and breast carcinomas. The glycosidic linkage of the Tn antigen, between *N*-acetylgalactosamine and serine or threonine, can be cleaved either chemically or enzymatically in the presence of glycosidases. The latter case is particularly a problem *in vivo*. Therefore, it would be of great interest to obtain a metabolically stable analogue of the Tn antigen that maintains or improves the immunogenic activity of the latter. The purpose of this work was to synthesize a totally synthetic vaccine using a chemically and metabolically stable glycomimetic of the Tn antigen in which the interglycosidic oxygen was replaced by a sulphur atom (*S*-Tn). The *S*-Tn thioglycopeptide was linked to the P_3CS immunoadjuvant to obtain the potential *S*-Tn vaccine. Moreover, we synthesized the natural Tn antigen and derivatized it similarly to obtain the Tn vaccine. Last, we evaluated the immunostimulating activity of the two synthetic potential vaccines *in vitro* using cultured mouse splenocytes. The *S*-Tn construct showed immunostimulating activity comparable, in terms of maximal response, to the Tn analogue. Moreover, due to its higher stability the *S*-Tn construct reached its maximal effect at lower doses compared to the Tn analogue.

INTRODUCTION

Cell-surface carbohydrates can undergo remarkable modifications as a result of malignant processes.[2] These alterations result from either incomplete glycosylation or neoglycosylation in tumor cells. Among the tumor associated carbohydrate antigens, Tn (GalNAcα1→O-Ser/Thr) and sialosyl Tn (NeuAcα2→GalNAcα1→O-Ser/Thr) are the most specific as they are basically absent from normal tissues (less than 1 out of 100,000 people).[3] Tn antigen is cryptic in normal cells. In fact, it is further glycosylated to constitute the core structure of mucin-type glycoproteins, whereas in most human carcinomas, in which the biosynthesis of carbohydrate chains is blocked, it is exposed at the cellular surface. The Tn antigen is expressed in over 70% of human epithelial cancers such as lung, colon, stomach and breast carcinomas.[4] It was also reported that this antigen had been identified as a partial structure of the HIV envelope glycoprotein gp120.[5] Therefore, carbohydrate antigens have generated considerable interest as potential targets for active specific immunotherapy (ASI) of cancer.[6] However, most carbohydrate antigens elicit only humoral responses.[7] To obtain an antigen-specific cellular immune response, it is important to have an efficient presentation of the synthetic carbohydrate to the immune system so that these antigens will be recognized by T and B lymphocytes. The Tn epitope was used[8] as a molecular component of a low molecular weight totally synthetic construct obtained by linking a dimeric Tn derivative to a synthetic lipopeptidic immunoadjuvant, tripalmitoyl-*S*-glyceryl-cysteyl-serine (P_3CS). The latter is a powerful B-cell and macrophage activator derived from the immunologically active *N*-terminal sequence of the lipoprotein obtained from the outer cellular membrane of *Escherichia coli* strains.[9]

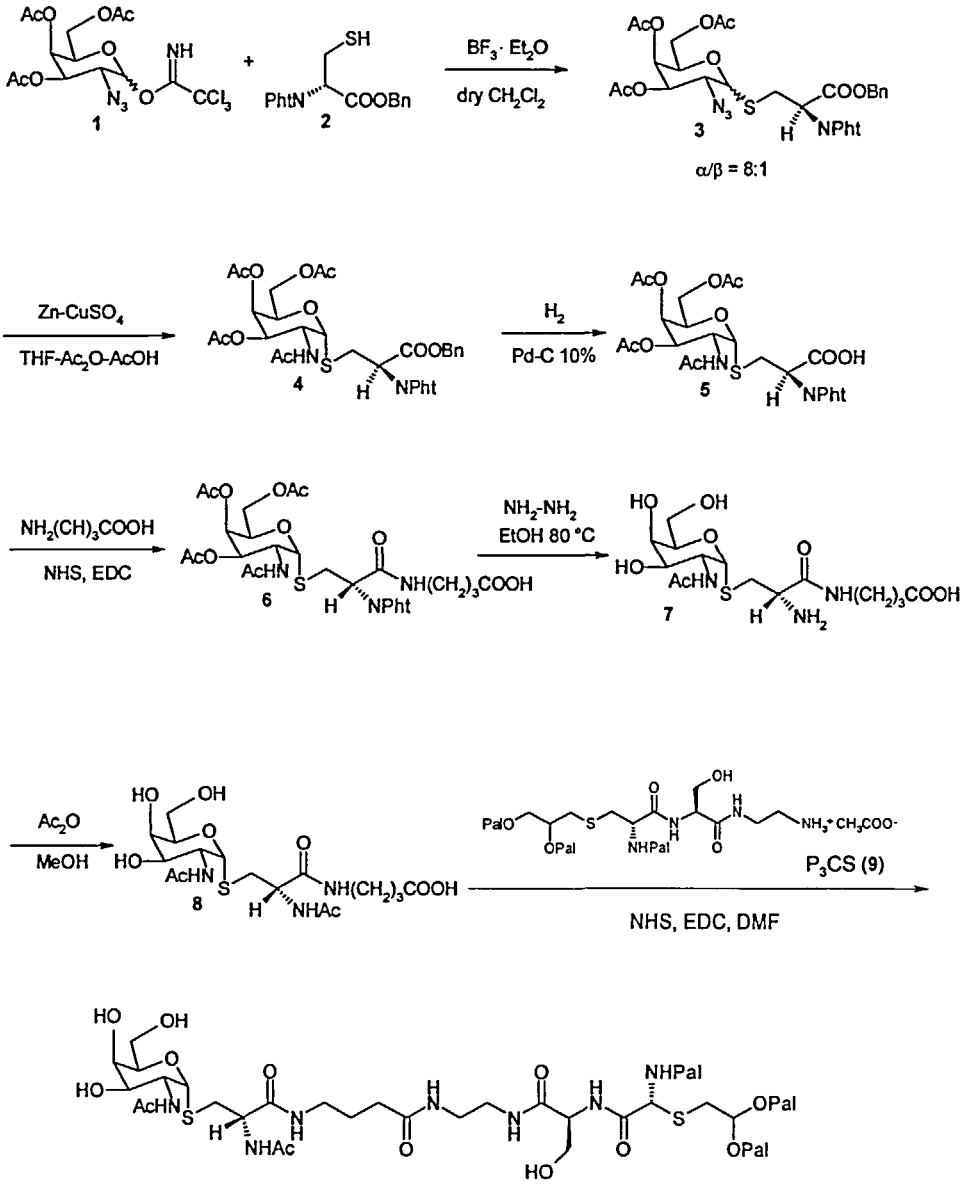
The development of new carbohydrate based drugs is often difficult due to the challenges associated with their synthesis and the relative instability of the glycosidic linkage, which is susceptible to acid and/or enzymatic hydrolysis. In fact, the glycosidic linkage of the Tn antigen, between *N*-acetylgalactosamine and serine or threonine, can be enzymatically cleaved *in vivo* by glycosidases. Glycomimetics have been developed to overcome such problems associated with limited stability. Our approach was to develop a thioglycosidic analogue of the Tn antigen as a stable glycomimetic which could maintain or even improve the biological activity of the natural derivative. Therefore, we prepared a totally synthetic construct **10** using an *S*-glycosidic derivative

of the Tn antigen (*S*-Tn, **8**) in which the interglycosidic oxygen was replaced by a sulphur atom, which for that reason, was anticipated to be metabolically more stable. Thus, the *S*-Tn antigen derivative was linked to the P_3CS immunoadjuvant to give the target **10**. We also synthesized the natural mono-Tn antigen (**11**),[8] which was then linked to the same immunoadjuvant to give the reference compounds **12**. Last, we compared the immunostimulating activity of the two synthesized potential vaccines *in vitro* using mouse splenocytes.

RESULTS AND DISCUSSION

It was reported that *S*-β-D-glucosylated and *S*-β-D-galactosylated L-cysteine derivatives were obtained by nucleophilic substitution using acetyl protected glycopyranosyl donors and urethane protected cysteine esters.[10,11] These methods gave exclusively the β-thioglycoside derivatives because of neighbouring-group participation. Alternatively, the *S*-β-D-glucosyl derivative can be obtained by alkylation of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose with a β-iodo-L-serine derivative. Schmidt and Stumpp[12] demonstrated that the acid catalysed reaction of 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate with simple thiols gave only *S*-α-D-glucosides because of the nonparticipating character of the group at C-2. Kasbeck and Kessler[13] attempted to use this reaction to obtain α-cysteine derivatives by glycosylation of *N*-benzyloxycarbonyl-L-cysteine benzyl esters with 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate, but failed because the urethane *N*-protecting group of the cysteine derivative reduced the nucleophilicity of the β-SH group. To avoid this problem, they used *N*-diphenylmethylene-protected cysteine esters and 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate in the presence of an equivalent of $BF_3{\cdot}Et_2O$. Under these conditions they obtained the desired *S*-glycoside derivative in an anomeric ratio of $\alpha/\beta = 5{:}1$.

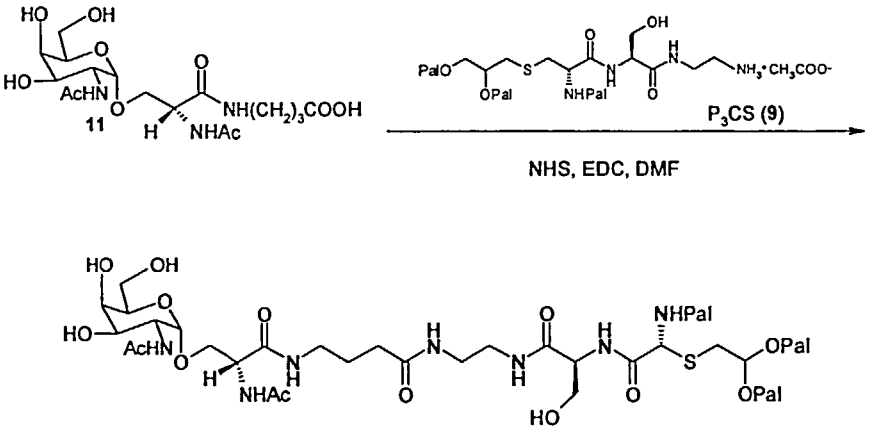
Therefore, we decided to use, for the synthesis of the metabolically stable *S*-analogue of the Tn antigen, the *N*-phthalyl-L-cysteine-benzyl ester (**2**)[13] and, as glycosyl donor, the 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl trichloroacetimidate (**1**).[14-20] They were condensed using $BF_3{\cdot}Et_2O$ as the promoter[13] (Scheme 1). The nonparticipating nature of the azido group, a precursor of the



Scheme 1. Synthesis of the *S*-Tn Vaccine

acetamido group, attached at C-2 of the glycosyl donor **1** ensured a good stereoselectivity of the thioglycosylation step resulting in thiogalactosyl derivative **3** as an α/β mixture. In fact, we obtained a 8.7:1.3 α/β ratio that was even better than that reported.[13] Next, the α/β mixture of **3** was converted into the corresponding 2-acetamido-2-deoxy-D-thioglycoside in one step by reducing the azido group with preactivated Zn powder in a 3/2/1 mixture of tetrahydrofuran, acetic anhydride and water.[21] The α-anomer **4** was separated by flash column chromatography from the β anomer. Selective hydrogenolysis[22] of the ester group of **4** yielded the galactosyl-cysteinyl derivative **5** bearing a free carboxylic group. Next, the 4-aminobutyric spacer, used to avoid any steric interferences with the carrier molecule P_3CS,[8] was introduced by means of NHS-carboxylic acid activation of the derivative **5** and subsequent treatment with 4-aminobutyric acid to give the galactosyl-4-aminobutanoic derivative **6**.[8] The removal of the *N*-phthaloyl and *O*-acetyl groups of the derivative **6**, was performed by hydrazinolysis to afford the amino derivative **7**.[13] Next, conventional selective acetylation[8] of the free amino group of **7** gave the derivative **8**. In the final step, the *S*-Tn antigen derivative **8** was coupled to the to P_3CS (**9**) using the NHS ester method[8] to give the *S*-Tn-construct (**10**). The P_3CS (**9**) (*N*-{*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(*2R-2S*)-propyl]-L-cysteyl-L-seryl}-1,2-diaminoethane) was prepared according to literature methods.[23]

The ^{1}H NMR spectra of the thioglycosides **6**, **7**, **8** and the *S*-Tn construct **10** showed a characteristic behaviour. In fact, some proton signals showed a typical splitting with respect to their parent derivative **5**, or the corresponding *O*-analogues. This trend arose as a consequence of the introduction of the aminobutyric spacer onto the thio-derivative **5**, and can be attributed to the presence at room temperature of a slow equilibrium between two different conformations of **6**, **7**, **8** and **10**, respectively. This fact could derive from hydrogen bonding between the NH-group of the spacer and the sulphur atom. This hypothesis was confirmed by ^{1}H NMR data for compound **6** obtained from spectra run at multiple temperatures. The two H-1 doublets, at 5.44 ppm and 5.42 ppm (Δ ppm = 0.02) observed at 27 °C, tended to approach each other as could be seen at 40 °C (5.57 ppm and 5.56 ppm, respectively with a Δ ppm = 0.01) and the two acetamido singlets, observed at 1.943 ppm and 1.939 ppm when the spectrum was run at rt, combined into a single singlet at 2.08 ppm when the spectrum is run at 40 °C.



Tn Vaccine (12)

Scheme 2. Synthesis of the Tn Vaccine

Analogously, two doublets in the range of 5.43-5.60 ppm were associated with the anomeric proton of the α-thioglycosides (**7**, **8** and **10**) which all bore the aminobutyric spacer. In contrast, for the thio-derivatives **3**, **4** and **5**, we observed as expected for the anomeric proton, a single doublet in the range of 5.58-5.62 ppm. All α-thioglycosides showed a typical $J_{1,2}$ coupling constant, in the range 5.4-5.6 Hz, that confirmed the C-1 α-configuration, whereas for β-thioglycosides larger coupling constant were reported ($J_{1,2} \approx 8$ Hz).[12,24-25]

The synthesis of the Tn-construct **12** was performed as described by Toyokuni[8] who linked a dimer of the Tn antigen to the P_3CS immunoadjuvant in order to obtain a totally synthetic vaccine that was able to stimulate antibody production in mice. Since we were interested to know if the *S*-Tn antigen derivative would maintain its immunogenic activity with respect to the natural Tn antigen, we simply coupled the mono Tn antigen derivative **11** to P_3CS (**9**) by the NHS ester method (Scheme 2), and used the resulting Tn construct as the reference compound in the pharmacological trials. The mono Tn antigen (*N*-[*N*-Acetyl-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl]-4-aminobutanoic Acid, **11**) was prepared according to literature methods[8] with

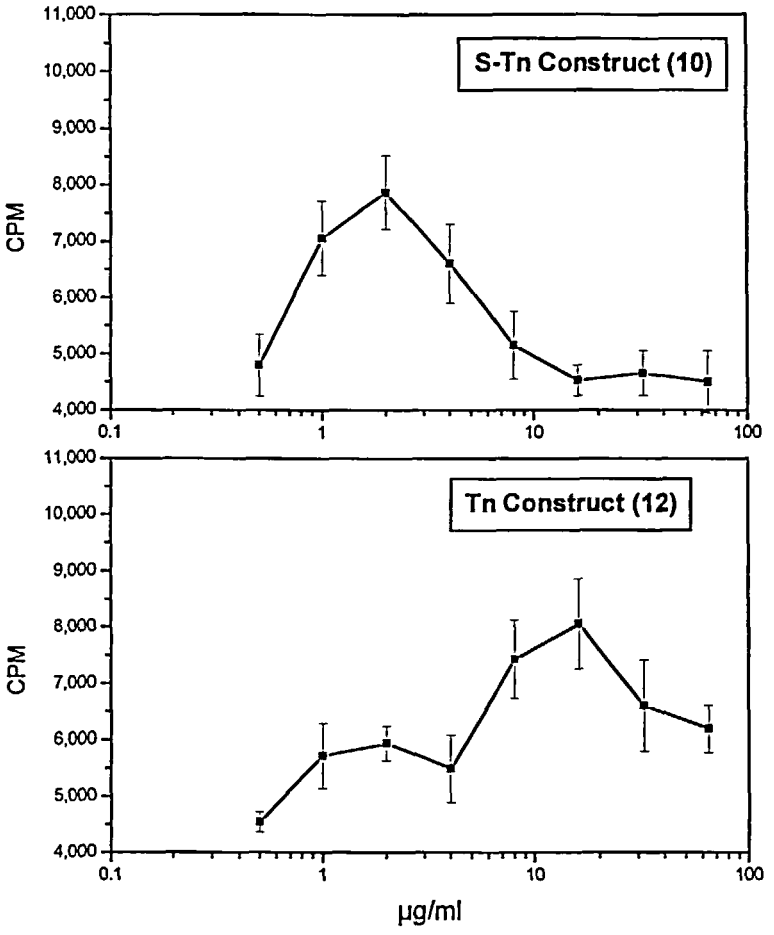


Figure 1. Dose-response curves for incorporation of [^{3}H] thymidine in Balb/c C57Bl/6 mouse splenocytes, after stimulation with *S*-Tn construct **10** and Tn construct **12**. The values for the incorporated radioactivity (CPM) represent the mean value of triplicate runs of three separate experiments.

the exception of the selective reduction of the azido group into the amino group and subsequent acetylation. This reaction was obtained by treatment with Zn powder, previously activated with 2% $CuSO_4$, of a solution of the azido derivative in a mixture of tetrahydrofuran, acetic anhydride and water (3/2/1).[21]

Both Tn and *S*-Tn constructs, **10** and **12**, were able to stimulate mice splenocytes proliferation in a concentration dependent manner (Fig. 1). The *S*-Tn construct **10** showed a stimulating activity comparable to that of the Tn construct **12**. Moreover, the

maximal effects were reached at concentrations of 16 and 2 μg/mL for **12** and **10**, respectively. This result indicated that **12** needed a dose 8-fold higher than that of **10** to ensure the same effect. The explanation of these results may reside in the higher chemical and enzymatic stability of the *S*-Tn construct **10** compared to the Tn analogue **12**. Thioglycosides exhibit exceptional chemical and enzymatic stability.[26] It was reported that the weaker basicity of the thioglycosides is the cause of their resistance to acid hydrolysis.[27,28] Moreover, because of the greater stability of the sulfonium ion with respect the oxocarbenium ion,[29] thioglycosides do not undergo spontaneous cleavage upon protonation, as glycosides do. The oxocarbenium ion formation is an important step in the catalytic process either for glycosidases that retain the anomeric configuration or for anomer-inverting enzymes.[30] Therefore, the lack of oxocarbenium ion formation in the thioglycosides supposedly caused the loss of the enzyme activity, and for this reason thioglycoside analogues of many carbohydrates are used as enzyme inhibitors.[31] We speculated that the different concentrations at which the maximal stimulation evoked by the two constructs was reached depended on their different stability. It is reasonable to suppose that the Tn vaccine reaches the maximum stimulation at higher doses with respect to the *S*-Tn antigen because of its lower chemical and enzymatic stability. After the maximal response was obtained both constructs became toxic.

The findings reported in this study demonstrated that the *S*-Tn construct **10** can act as a chemically and enzymatically stable glycomimetic of the Tn construct **12** that retained its immunostimulating activity in the mouse splenocytes. In conclusion, the *S*-Tn construct **10** could be reasonably proposed as a candidate for immunostimulation *in vivo*. Studies are in progress to investigate the possible anticancer activity of the *S*-Tn construct in mice. Experiments to confirm the metabolic stability in plasma of both *S*-Tn and Tn constructs are currently being performed.

EXPERIMENTAL SECTION

General Methods. ^{1}H and ^{13}C NMR spectra were measured on Varian Unity Inova 200 MHz and Varian Unity Inova 500 MHz spectrometers with TMS in organic solvent and DSS in D_2O as internal standards. Elemental analyses for C, H and N were

carried out using an elemental analyser, Carlo Erba Model EA 1108. Optical rotations were recorded on an Optical Activity AA-10 automatic polarimeter in a 5 cm cell at 21 °C. Flash column chromatography was performed as reported[32] using silica gel (mesh size 0.040-0.063 mm, Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on Merck (Darmstadt, Germany) silica gel plates 60 F_{254}. The detection of the spots in the TLC plates were performed by UV-detection or using the following spray reagents: 10% H_2SO_4 in ethanol for saccharide compounds; ninhydrin reagent for compounds with a primary amino group; a) 0.1% fluorescein in ethanol 50%, b) 30% H_2O_2 in CH_3COOH for compounds with bromine; 0.1 % sodium nitroprusside in ethanol solution for compounds containing an -SH group; a) 50% NaClO, b) ethanol, c) KI in starch paste for amide compounds. Anhydrous acetonitrile, dichloromethane, chloroform and dimethylformamide were prepared according to the standard procedures. All other solvents and chemicals were reagent grade and purchased from Sigma, Aldrich or Fluka (Milano, Italy).

***N*-Phthalyl-3-*S*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α,β-D-galactopyranosyl)-L-cysteine Benzyl Ester (3).** A soln of glycosyl trichloroacetimidate[14-20] **1** (α/β = 1:1, 274.5 mg, 0.577 mmol) and of *N*-phthalyl-L-cysteine benzyl ester[13] (**2**, 194.8 mg, 0.571 mmol) in anhydrous dichloromethane under nitrogen was stirred for 30 min and 71.6 μL of $BF_3{\cdot}Et_2O$ were added. After 2 h at rt, the reaction mixture was neutralised by adding 79.2 μL of triethylamine, and extracted three times with water. The organic layer was dried ($MgSO_4$) and concentrated to give a yellowish oil. Purification by flash column chromatography on silica gel (hexane/ethyl acetate 2/1) gave 196 mg (52.4%) of **3** as an anomeric mixture (α/β = 8:1 as determined from ^{1}H NMR data). ^{1}H NMR ($CDCl_3$) δ 7.91-7.85 (m, 4H, phthalyl), 7.33 (m, 5H, phenyl), 5.53 (d, 1H, $J_{1,2}$ = 5.6 Hz, H-1 α-sugar), 5.40 (dd, 1H, $J_{4,5}$ = 0.5 Hz, H-4), 5.21, 5.19 (2s, 2H, -OCH_2Ph), 5.09 (dd, 0.87 H, $J_{\alpha,\beta'}$ = 4.8 Hz, $J_{\alpha,\beta}$ = 10.6 Hz, α-H), 4.99 (dd, 1H, $J_{2,3}$ = 11.2 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 4.60 (t, 1H, $J_{5,6}$ = 6.0 Hz, H-5), 4.41 (d, 0.13 H, $J_{1,2}$ = 12.3 Hz, H-1 β-sugar), 4,24-4.12 (m, 3H, H-2, 2H-6), 3.60 (dd, 1H, $J_{\beta,\beta'}$ = 14.2 Hz, β-H), 3.47 (dd, 1H, β'-H), 2.14, 2.01, 1.94 (3s, 9H, OAc).

Anal. Calcd for $C_{30}H_{30}O_{11}N_4S$: C, 55.04; N, 8.56; H, 4.59. Found: C, 55.09; N, 8.61; H, 4.52.

***N*-Phthaloyl-3-*S*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-cysteine Benzyl Ester (4).** A soln of **3** (126.2 mg, 0.193 mmol) in

tetrahydrofurane/acetic anhydride/water (3/2/1) was treated with 133.3 mg of Zn powder previously activated with 2% $CuSO_4$ and washed three times with water. The reaction mixture was stirred for 3 h at room temperature and then was filtered through Celite and concentrated. Purification by flash column chromatography on silica gel (toluene/ethyl acetate 1/2) gave 60 mg of **4** (46.5 %) as a white foam. ^{1}H NMR ($CDCl_3$) δ 7.91-7.75 (m, 4H, phthalyl), 7.33 (m, 5H, phenyl), 5.58 (d, 1H, $J_{1,2}$ = 5.4 Hz, H-1), 5.53 (d, 1H, $J_{NH,H-2}$ = 8.8 Hz, NHAc), 5.36 (dd, 1H, $J_{4,5}$ = 1.4 Hz, H-4), 5.19, 5.18 (2 s, 2H, OCH_2Ph), 5.08 (dd, 1H, $J_{\alpha,\beta'}$ = 4.4 Hz, $J_{\alpha,\beta}$ = 10.8 Hz, CysαCH), 4.95 (dd, 1H, $J_{2,3}$ = 11.8 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 4.71 (ddd, 1H, H-2), 4.56 (t, 1H, $J_{5,6}$ = 6.8 Hz, H-5), 4,26-4.13 (m, 2H, 2H-6), 3.67 (dd, 1H, $J_{\beta,\beta'}$ = 14.2 Hz, Cysβ-H), 3.43 (dd, 1H, β'-H), 2.14, 1.97, 1.95, 1.85 (4 s, 12H, -NAc, -OAc).

Anal. Calcd for $C_{32}H_{34}O_{12}N_2S$: C, 57.31; N, 4.18; H, 5.07. Found: C, 57.29; N, 4.25; H, 5.03.

***N*-(Phthalyl)-3-*S*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-cysteine (5).** Compound **4** (120 mg, 0.179 mmol) was dissolved in 5 mL of a mixture of acetic acid and methanol (4/1) under nitrogen. The catalyst (30 mg of 10% Pd/C) was added, and the mixture was stirred under an H_2 atmosphere at rt for 3 h. The mixture was filtered and the filtrate was concentrated in vacuo to provide quantitatively the derivative **5** as a light yellow oil. ^{1}H NMR (CD_3OD) δ 7.94 -7.83 (m, 4H, phthalyl), 5.62 (d, 1H, $J_{1,2}$ = 5.4 Hz, H-1 of α-sugar), 5.42 (dd, 1H, $J_{4,5}$ = 1.0 Hz, H-4), 4.64 (t, 1H, $J_{5,6}$ = 6.8 Hz, H-5), 4.51 (dd, 1H, $J_{2,3}$ = 12.2 Hz, H-2), 4,21-4.07 (m, 3H, 2H-6, β-H), 3.62 -3.42 (m, 1H, β'-H), 2.11, 2.04, 1.99, 1.91 (4 s, 12H, NAc, OAc).

Anal. Calcd for $C_{25}H_{28}O_{12}N_2S$: C, 51.72; N, 4.82; H, 4.83. Found: C, 51.63; N, 4.88; H, 4.79.

***N*-[*N*-Phthalyl-3-*S*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-cysteyl]-4-aminobutanoic Acid (6).** Compound **5** (86.6 mg 0.149 mmol), NHS (25.4 mg, 0.22 mmol) and EDC (42.5 mg, 0.22 mmol) were dissolved in anhydrous dichloromethane (4 mL) under nitrogen. The reaction mixture was stirred at rt for 1 h, and afterward was extracted three times with cold water. The organic layer was dried ($MgSO_4$) and concentrated to give 72.8 mg (73.9 %) of the *N*-(phthalyl)-3-*S*-(3,4,6-tri-*O*-acetyl-2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-cysteine succinimido ester that was further used without purification. A pre-cooled soln of aminobutyric acid

(11.3 mg, 0.109 mmol) and DIPEA (16.7 μL, 0.099 mmol) in 0.50 mL of water was added dropwise under nitrogen to a soln of the succinimido ester derivative of **5** (64.4 mg, 0.097 mmol) in dry DMF at 0 °C, and the mixture was stirred at 0 °C for 1 h. After treatment with Amberlite® IR 120 (H^+) resin until neutralisation, the mixture was filtered and the filtrate was concentrated in vacuo. Purification by LH-20 column chromatography (acetone) yielded the derivative **6** (60.2 %). ^{1}H NMR ($CDCl_3$) δ 7.85 - 7.72 (m, 4H, phthalyl), 5.95 (d, 0.36 H, J = 8.4 Hz, NHAc), 5.77 (d, 0.64 H, J = 8.4 Hz, NHAc), 5.60 (2d (see text), 1H, $J_{1,2}$ = 5.4 Hz, H-1), 5.32 (m, 1H, H-4), 4.83-4.90 (m, 2H, H-3, CysαCH), 4.68 (m, 1H, H-2), 4.43 (t, 1H, J = 6.4 Hz, H-5), 4.14 (d, 1H, J = 6.4 Hz, H-6a), 4.06 (d, 1H, J = 6.4 Hz, J = 3.2 Hz, H-6b), 3.40-3.20 (m, 2H, CysβCHa, CysβCHb), 3.20-2.80 (br m, 2H, $NCH_2CH_2CH_2CO$), 2.36 (br t, 2H, J = 6.0 Hz, $NCH_2CH_2CH_2CO$), 2.14 (d, 3H, J = 5.0 Hz, NHAc), 2.04, 1.94, 1.86, (3s, 12H, 3-OAc), 1.85 (br quintet, 2H, J = 6.0 Hz, $NCH_2CH_2CH_2CO$).

Anal. Calcd for $C_{29}H_{35}O_{13}N_3S$: C, 52.33; N, 6.32; H, 5.26. Found: C, 52.41; N, 6.29; H, 5.32.

***N*-[3-*S*-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-L-cysteyl]-4-aminobutanoic Acid (7).** Compound **6** (64.4 mg, 0.097 mmol) together with 2.5 mL of hydrazine hydrate were dissolved in 40 mL of ethanol and refluxed for 2 h. The reaction mixture was concentrated in vacuo, and purification by flash column chromatography on silica gel (chloroform/methanol 1/4) gave 21.3 mg of **7** as a colourless oil (54 %). ^{1}H NMR (D_2O) δ 5.43 (2d, 1H, $J_{1,2}$ = 5.5 Hz, H1), 4.26 (m, 1H, CysαCH), 4.17 (m, 1H, H-2), 3.89 (t, 1H, $J_{3,4}$ = 4 Hz, H-4), 3.76-3.68 (m, 3H, H-5, 2H-6), 3.59 (2dd, 1H, $J_{2,3}$ = 11 Hz, H-3), 3.20-3.00 (br m, 2H, $NCH_2CH_2CH_2CO$), 2.88, 2.75 (2ddd, 2H, CysβCHa, CysβCHb), 2.13 (2t, 2H, J = 6.5 Hz, $NCH_2CH_2CH_2CO$), 1.94 (d, 3H, J = 2.0 Hz, NHAc), 1.68 (quintet, 2H, J = 6.5 Hz, $NCH_2CH_2CH_2CO$).

Anal. Calcd for $C_{15}H_{27}O_8N_3S$: C, 44.01; N, 10.27; H, 6.60. Found: C, 43.94; N, 10.12; H, 6.71.

***N*-[*N*-Acetyl-3-*S*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-cysteyl]-4-aminobutanoic Acid (S-Tn Antigen) (8).** Compound **7** (21.3 mg, 0.052 mmol) was dissolved in 2.5 mL of methanol, and 11.8 μL of acetic anhydride was added and the mixture was stirred at rt for 2 h. The reaction mixture was subsequently concentrated in vacuo and redissolved in 4.5 mL of a 2:1 methanol/water mixture. Sodium hydroxide

(0.45 mL, 0.1 M) was added and the mixture stirred at rt for 15 min. After neutralisation with Amberlite® IR 120 (H^+) resin, the mixture was filtered, and the filtrate was concentrated in vacuo. Purification by Biogel P-2 column chromatography (water) yielded 21.4 mg (91.1 %) of derivative **8** as a white amorphous solid. ^{1}H NMR (D_2O) δ 5.45 (2d, 1H, $J_{1,2}$ = 5.5 Hz, H-1), 4.26 (m, 1H, CysαCH), 4.4 (m, 1H, H-3), 4.17 (m, 1H, H-2), 3.89 (t, 1H, H-4, $J_{3,4}$ = 3.5 Hz), 3.76-3.68 (m, 3H, H-5, 2H-6), 3.20-3.00 (br m, 2H, $NCH_2CH_2CH_2CO$), 2.88, 2.75 (2ddd, 2H, CysβCHa, CysβCHb), 2.26 (br t, 2H, J = 6.5 Hz, $NCH_2CH_2CH_2CO$), 1.95, 1.94 (2d, 6H, J = 6.0 Hz, J = 3.5 Hz, 2 NHAc), 1.7 (br quintet, 2H, J = 6.5 Hz, $NCH_2CH_2CH_2CO$).

Anal. Calcd for $C_{17}H_{29}O_9N_3S$: C, 45.23; N, 9.31; H, 6.43. Found: C, 45.28; N, 9.36; H, 6.37.

***N*-{*N*-Palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(*2R-2S*)-propyl]-L-cysteyl-L-seryl}-2-aminoethyl-*N*^{4}-[*N*-acetyl-*S*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl-4-aminobutanamide (*S*-Tn-construct) (10).** A solution of **8** (20 mg, 0.044 mmol), NHS (5.4 mg, 0.047 mmol) and EDC (8.9 mg, 0.047 mmol) in anhydrous dimethylformamide (0.7 mL) was stirred at rt. The reaction was monitored by TLC (chloroform/methanol 1/1) until one major product (R_f = 0.59) was formed (4 h). At this time was added dropwise a solution of **9** (47.3 mg), DIPEA (8.1 µL, 0.046 mmol) in 2.0 mL of anhydrous dimethylformamide, and the mixture was stirred at rt for 1 h. The reaction mixture was concentrated in vacuo and washed three times with water. The solid obtained was freeze-dried to yield 40 mg (70 %) of a white amorphous solid corresponding to the *S*-Tn construct (**10**). $[\alpha]_D$ + 4.9 (*c* 0.30, DMF); ^{1}H NMR ($CDCl_3/CD_3OD/D_2O$ 6.5/2.5/0.4) δ 5.53, (2d, 1H, $J_{1,2}$ = 5.5 Hz, H-1), 5.18 (m, 1H, CHOCO), 4.60 (m, 1H, CysαCH), 4.38 (m, 1H, H-2), 3.41-3.24 (m, 4H, NCH_2CH_2N), 3.20 (br t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.08-3.00 and 2.94-2.83 (2 m, 4H, 2 Cysβ-CH_2), 2.77 (m, 2H, SCH_2CHOCO), 2.34, 2.32, 2.28 (3 br t, 6H, J = 7.5 Hz, 3 PalCH_2CO), 2.20 (br t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 2.03, 2.01 (2d, 6H, 2 NAc), 1.77 (br quintet, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 1.62, 1.28 (2 m, 39 Pal-CH_2), 0.89 (t, 9H, J = 6.5 Hz, 3 Pal-CH_3).

Anal. Calcd for $C_{76}H_{141}O_{16}N_7S_2$: C, 61.96; N, 6.66; H, 9.65. Found: C, 61.89; N, 6.71; H, 9.69.

***N*-{*N*-Palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(*2R-2S*)-propyl]-L-cysteyl-L-seryl}-2-aminoethyl-{*N*^{4}-[*N*-acetyl-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl**

aminobutanamide} (Tn-vaccine) (12). A solution of **11** (20 mg, 0.046 mmol), NHS (5.41 mg, 0.047 mmol) and EDC (9.3 mg, 0.049 mmol) in anhydrous dimethylformamide (0.7 mL) was stirred at rt. The reaction was monitored by TLC (chloroform/methanol 1/1) until one major product (R_f= 0.59) was formed (4 h). At this time was added dropwise a solution of 9 (47.8 mg), DIPEA (8.5 μL, 0.048 mmol) in 2.0 mL of anhydrous dimethylformamide and the mixture was stirred at rt for 1 h. The reaction mixture was concentrated in vacuo and washed three times with water. The solid obtained was freeze-dried to yield 40 mg (70 %) of a white amorphous solid of the Tn construct (**12**). $[\alpha]_D$ + 1.3 (*c* 0.29, DMF); ^{1}H NMR ($CDCl_3/CD_3OD/D_2O$ 6.5/2.5/0.4) δ 5.18 (m, 1H, CHOCO), 4.83 (br d, 1H, $J_{1,2}$ = 4.0 Hz, H-1), 3.90 (br d, 1H, $J_{3,4}$ = 3.0 Hz, H-4), 3.41-3.24 (m, 4H, NCH_2CH_2N), 3.20 (br t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 3.08-3.0 e 2.94-2.83 (2 m, 2H, Cysβ-CH_2), 2.77 (m, 2H, SCH_2CHOCO), 2.34, 2.32, 2.28 (3 br t, 6H, J = 7.5 Hz, 3 PalCH_2CO), 2.20 (br t, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 2.04, 2.03 (2s, 6H, 2 NAc), 1.77 (br quintet, 2H, J = 7.0 Hz, $NCH_2CH_2CH_2CO$), 1.62, 1.28 (2 m, 39 Pal-CH_2), 0.89 (t, 9H, J = 6.5 Hz, 3 Pal-CH_3).

Anal. Calcd for $C_{76}H_{141}O_{17}N_7S$: C, 62.64; N, 6.73; H, 9.76. Found: C, 62.73; N, 6.78; H, 9.82.

Lymphocyte cultures. The immunostimulating activity of the glycolipopeptides was evaluated in C57Bl/6 mouse splenocytes *in vitro*. Spleens were aseptically removed and mechanically dissociated to a single cell suspension by pressing with the blunt end of a 5 mL syringe plunger in a complete medium. Both peripheral blood lymphocytes and splenocyte suspension were then stratified onto a Ficoll-Hypaque gradient (Pharmacia, Piscataway, NJ) and centrifuged for mononucleate cell separation. The resulting buffy coat was then washed twice in PBS, the cells counted and plated in 96-well plastic microplates (Costar, Cambridge, MA, USA) at the density of 2 x 10^5/100 μL/well. Concanavalin A (ConA) was added in 100 μL graded concentrations. Cells were incubated for 72 h at 37 °C in 5% CO_2-saturated atmosphere. Then, cells were incubated for 6 h with 1 μCi/well of tritiated thymidine [^{3}H]-THY (New England Nuclear Florence, Italy; volume 20 μL/well) and harvested onto fiberglass filters (Skatron, Noway). Heath-dried filters were placed in plastic tubes and, after the addition of 3.5 mL of scintillating solution (Instagel, Beckman), radioactivity was counted for 2

min in a β-counter. Each sample was run in triplicate. Experiments were repeated at least twice.

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