Bioconjugate Chemistry

Article

pubs.acs.org/bc

First Self-Adjuvant Multicomponent Potential Vaccine Candidates by Tethering of Four or Eight MUC1 Antigenic Immunodominant PDTRP Units on a Calixarene Platform: Synthesis and Biological Evaluation

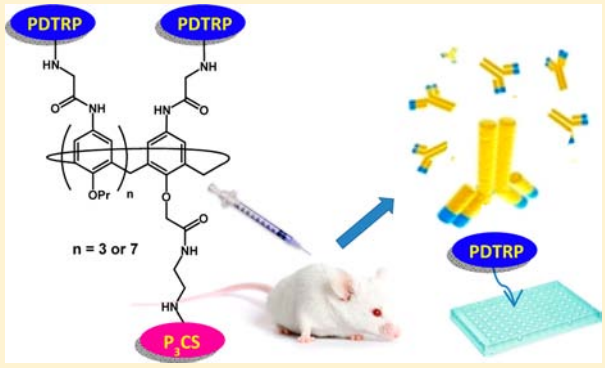
Corrada Geraci,*[,†] Grazia M. L. Consoli,[†] Giuseppe Granata,[†,‡] Eva Galante,[†] Angelo Palmigiano,[§] Maria Pappalardo,[‡] Salvatore D. Di Puma,[‡] and Angelo Spadaro*[,‡]

[†]CNR Istituto di Chimica Biomolecolare, Via P. Gaifami 18, I-95126 Catania, Italy

[§]CNR Istituto per la Chimica e la Tecnologia dei Polimeri, Via P. Gaifami 18, I-95126 Catania, Italy

[‡]Dipartimento di Scienze del Farmaco, Università di Catania, Viale A. Doria 6, I-95125 Catania, Italy

ABSTRACT: MUC1 protein overexpressed in human epithelial carcinoma is a target in development of novel anticancer vaccines. Multiple units of immunodominant B-cell epitope PDTRP MUC1 core sequence were conjugated to calix[4,8]arene platforms containing TLR2 ligand, to produce two novel anticancer self-adjuvant vaccine candidates. The immunogenicity of the synthetic constructs was investigated by immunization of mice in vivo. ELISA assay evidenced that the vaccine candidates stimulate anti MUC1 IgG antibody production (major for the octavalent construct) and no additive effect but a multivalency effect was observed when compared to an analogous monovalent. Octa- and tetravalent constructs lacking in PDTRP peptide moieties did not show anti MUC1 IgG antibody production in mice. The antibodies induced by the synthesized constructs are able to recognize the MUC1 structures present on MCF7 tumor cells. The results display that calixarenes are convenient platforms for building multicomponent self-adjuvant vaccine constructs promising as immunotherapeutic anticancer agents.



INTRODUCTION

Tumor immunotherapy, as confirmed by profuse literature in the last 10−15 years, is an intriguing approach for the treatment of cancer. This is based on the use of functional antitumor vaccine able to elicit a specific immune reaction on tumor cell without affecting healthy tissue.[1]

In the prevalent epithelial tumors, such as breast, pancreatic, lung, colorectal, and ovarian cancer, more information about the structural elements to distinguish tumor cells from normal cells is available. In particular, MUC1, the large transmembrane glycoprotein present at the apical surface of the normal glandular epithelial cell, is overexpressed in the tumor-associate state, and it attracts real interest as a potential target in the development of vaccine for tumor immunotherapy.[2] The MUC1 extracellular domain is formed by tandem repeats comprising 20 amino acid sequence (GVTSA**PDTRP**APGSTAPPAH)$_n$ where n varies between 30 and 100, so that the extracellular domain of this glycoprotein may extend 150−500 nm above the cell membrane.[3] Different numbers of tandem repeats are also the cause of the polymorphism exhibited by MUC1.[4]

In normal mucin, the extracellular domain is densely covered with highly branched complex carbohydrate structures. There are five potential *O*-glycosylation sites in each peptide tandem repeat of the MUC1 corresponding to the serine and threonine residues, and the position of the glycosylation may vary depending on the kind of tissue. In the tumor-associate state, an aberrant glycosylation leads to the increased exposure of immunodominant areas and MUC1 becomes an autoantigen; in fact, the underglycosylation determines the exposure of normally cryptic tumor-associated carbohydrate antigens (i.e., Tn, STn, TF, etc.), as well as of a highly immunogenic PDTRP core peptide sequence.[5] The PDTRP sequence has been identified as the immunodominant B-cell epitope from monoclonal antibody studies in mice and it is also believed to be immunodominant in humans. In breast cancer patients, MUC1-expressing tumors develop humoral and cellular immune responses to the PDTRP sequence.[6]

Recently, we realized a self-adjuvant tetravalent vaccine candidate by clustering four *S*-Tn tumor associated glycomimetic antigens and incorporating a Toll-like receptor 2 (TLR2) ligand on a "nonpeptidic" calix[4]arene platform.[7] This construct, with definite spatial preorganization of the antigenic moieties, elicited a higher specific imumostimulating activity in mice than the monovalent analogous compound,[8] denoting a multivalency effect. It is known that multivalent binding

Received: May 16, 2013
Revised: July 29, 2013
Published: August 22, 2013

interactions are both kinetically and thermodynamically more efficient than a single interaction.[9]

With these results in hand, we decided to design two novel vaccine candidates by tethering for the first time four or eight PDTRP MUC1 core sequences on a calix[4,8]arene skeleton, respectively. To enforce the humoral immune response the known immunoadjuvant tripalmitoyl-*S*-glyceryl-cysteinylserine (P_3CS) was also introduced in the planned constructs. The P_3CS moiety, derived from the N-terminal sequence of the principal *Escherichia coli* lipoprotein, elicits its activity through the interaction with Toll-like receptor 2 (TLR2),[10−13] and determines the production of cytokines and chemokines which stimulate the antigen presenting cells with development and activation of T helper cells.

Biological tests performed *in vivo* by immunization of mice showed that the target constructs induce the production of anti MUC1 IgG antibodies and the vaccine candidate built on a conformational mobile calix[8]arene skeleton possesses a higher immunostimulating activity. As expected for compound lacking in PDTRP peptide moieties, no anti MUC1 IgG antibody production in mice was observed, indicating that calixarene platforms are convenient carriers for vaccine constructs.

EXPERIMENTAL PROCEDURES

General Considerations. Commercially purchased solvents and reagents were all of reagent quality. Analytical TLCs were performed on 0.25 mm silica gel 60 coated aluminum foils with F-254 indicator (Merck). Preparative TLC were performed on silica gel (Merck). Column chromatography (LC) was performed on silica gel 60 (0.040−0.063 mm, Merck) or on silica gel 100 C18-RP (230−400 mesh, Fluka). Size exclusion column chromatography was carried out on Sephadex LH-20 (Sigma-Aldrich). ^{1}H NMR (400.13 MHz), ^{13}C NMR (100.61 MHz), and 2D NMR spectra were acquired on a Bruker Avance 400 spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm). Spectra were referenced to the residual proton solvent peaks; coupling constant (J) values are given in Hz. Mass spectra were recorded on a mass spectrometer Waters Micromass ZQ 2000 model ESI-MS (positive ion mode) or on a MALDISTR Applied Biosystems instrument (positive ion, linear mode, accelerating voltage 24000, 256 laser shot). Microplates were read in a Wallac 1420 Victor2 plate reader (Perkin-Elmer).

Syntheses and Structural Characterization. Starting compound **1**[7] and P_3CS derivative[8,11] were prepared following the procedures previously reported.

*Tetra-Protected PDTRP-Gly-C4-P_3CS (**3**).* The fully protected pentapeptide PDTRP (**2**) (60 mg, 0.053 mmol) was dissolved in anhydrous DMF (4 mL) and reacted with PyBOP (39 mg, 0.074 mmol) and DIPEA (26 μL, 0.15 mmol). After stirring for 20 min, a solution of **1** (20 mg, 9.7 μmol) and DIPEA (26 μL, 0.15 mmol) in anhydrous DMF (4 mL) was added. The mixture was kept under stirring, at room temperature overnight. After evaporation of the solvent, the residue was suspended in 0.1 N HCl, collected by filtration, dried (Na_2SO_4), and purified by preparative TLC (CH_2Cl_2/MeOH 92:8, v/v) to give pure compound **3** as a white powder (37 mg, 60%). Because of the overlap of many signals in the ^{1}H NMR spectrum, we report the chemical shift values of some diagnostic signals. ^{1}H NMR ($CDCl_3$/MeOD 4:1 v/v, 297 K) δ 0.79 (t overlapped, J = 7.0 Hz, 12H, propyl CH_3 and 3 × palmitoyl CH_3), 0.87 (br t, 6H, 2 × propyl CH_3), 1.00 (br s, 12H, 4 × thr CH_3), 1.07 (br s, 36H, 4 × thr *tert*-Bu), 1.18 (br s, 72H, 36 × palmitoyl CH_2), 1.30 (s, 36H, 4 × aspartate *tert*-Bu), 1.52 (m, 6H, 3 × palmitoyl CH_2CH$_3$), 1.75 (overlapped 6H, 3 × propyl CH_2CH$_3$), 2.22 (m, J = 7.0 Hz, 6H, 3 × palmitoyl CH_2CO), 2.60 and 2.70 (br m, 1H each, cysβ-CH_2), 2.67 (br m, 2H, SCH_2CHOCO), 2.98 and 4.25 (br d, 2H each, ArCH_2Ar), 3.02 and 4.27 (br d, 2H each, ArCH_2Ar), 3.50 and 3.63 (t, J = 4.4 Hz, 2H each, NCH_2CH_2N), 3.71 (br m, 6H, 3 × propyl OCH_2), 4.24 (br m, 4 × Fmoc CH), 4.63 (br m overlapped, 1H, cysα-CH), 5.15 (br m, 1H, SCH$_2$CHOCO), 6.40 (br s, 4H, calixarene ArH), 6.44 (s, 4H, 4 × Mtr CH), 7.15 (br s, 4H, calixarene ArH), 7.22 (br m, 8H, 8 × Fmoc ArH), 7.31 (br m, 8H, 8 × Fmoc ArH), 7.50 (br d, 8H, 8 × Fmoc ArH), 7.68 (br d, 8H, 8 × Fmoc ArH). ^{13}C NMR ($CDCl_3$/MeOD 4:1, 297 K): 11.5, 13.6, 17.9 (q), 22.3 (t), 23.6 (q), 24.2, 24.7, 25.3, 25.9, 26.0 (t), 27.4, 27.7 (q), 28.8, 29.0, 29.3, 31.1, 31.6, 33.8, 34.0, 35.8, 36.2, 39.9, 42.9, 45.9, 46.7 (t), 46.8 (d), 47.3 (t), 49.3, 49.6, 50.4 (d), 53.1 (t), 55.0, 57.8 (d), 60.0 (q), 60.3 (d), 60.9, 63.4 (t), 66.1 (d), 67.6 (t), 69.9 (d), 72.0 (t), 74.7 (s), 77.2 (d), 81.5 (s), 111.4 (d), 119.6 (d), 124.4 (s), 124.7 (d), 126.8 (d), 127.4 (d), 133.3, 136.2, 138.1, 141.0, 143.4, 143.6, 155.5, 156.3, 158.1, 170.4, 172.6, 172.8, 172.9, 173.3, 173.5 (s).

*Tetra-PDTRP-Gly-C4-P_3CS (**4**).* Compound **3** (35 mg, 5.4 μmol) was stirred with piperidine (20% DMF solution, 0.5 mL) at room temperature for 2 h. After removal of the solvent under vacuum, the residue was dissolved in EtOH/CH_2Cl_2 mixture and evaporated, and after in Et_2O/CH_2Cl_2 mixture and evaporated. The crude product was purified by column chromatography on Sephadex-LH20 (100% CH_2Cl_2) to give pure de-Fmoc derivative (20 mg, 67%) as confirmed by the disappearance of the Fmoc signals in the ^{1}H NMR spectrum. To remove the remaining protective groups, the de-Fmoc derivative (19 mg, 0.0034 mmol) was treated with a mixture (1.3 mL) of trifluoroacetic acid, thioanisol, phenol, triisopropylsilane, thiocresol, and H_2O (8:0.5:0.3:0.25:0.1:0.5, v/v). The mixture was kept under stirring, at room temperature for 24 h. The solvent was removed under vacuum and the residue was suspended in Et_2O/EtOAc mixture and evaporated to give a solid residue. Pure compound **4** (11.2 mg, 79% yield) was obtained after column chromatography on Sephadex-LH20 (CH_2Cl_2/MeOH 9:1, v/v). The removal of the protective groups was confirmed by the disappearance of the proton resonances relative to Mtr and *tert*-Bu groups in the ^{1}H NMR spectrum. MALDI-TOF m/z calcd for $C_{202}H_{325}N_{44}O_{49}S$ $[M+H]^+$ 4186.1; found m/z 4188.3; m/z calcd for $C_{202}H_{324}N_{44}NaO_{49}S$ $[M+Na]^+$ 4208.1; found m/z 4205.4; m/z calcd for $C_{202}H_{324}N_{44}KO_{49}S$ $[M+K]^+$ 4224.2; found m/z 4221.3.

*5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55-heptakis[(4-bromobenzoyl)oxy]calix-[8]-aren-56-ol (**5**).* **5** was prepared by the procedure reported previously by our group.[14]

*5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55-heptakis[(4-bromobenzoyl)oxy]-56-[ethoxy-carbonyl-methoxy]-calix[8]arene (**6**).* A solution of **5** (3.20 g, 1.24 mmol) in anhydrous DMF (40 mL) was reacted with $BrCH_2COOEt$ (275 μL, 2.48 mmol) in the presence of Cs_2CO_3 (1.21 g, 3.71 mmol), under stirring at room temperature for 3 h. DMF was removed under vacuum, and the solid residue was triturated with 0.1 N HCl. The insoluble material was collected by filtration, washed with water and MeOH, and dried to give compound **6** (2.97 g, 90%) as a white

powder. ^{1}H NMR (C_6D_6, 355 K): δ 0.74 (t, J = 7.2, 3H, CH_2CH_3), 1.06 (s, 9H, *tert*-Bu), 1.10 (s, 54H, 6 × *tert*-Bu), 1.15 (s, 9H, *tert*-Bu), 3.73 (q, J = 7.2, 2H, CH_2CH_3), 3.88 (s, 4H, 2 × $ArCH_2Ar$), 3.90 (s, 4H, 2 × $ArCH_2Ar$), 3.96 (s, 4H, 2 × $ArCH_2Ar$), 4.11 (s, 6H, 2 × $ArCH_2Ar$ e OCH_2), 7.14−7.28 (overlapped, 30H, 16 × ArH e 14 × ArH-Bz), 7.50 (d, 2H, J = 7.6 Hz, ArH-Bz), 7.51 (d, 4H, J = 7.6 Hz, ArH-Bz), 7.60 (d, J = 8.1 Hz, 4H, ArH-Bz), 7.72 (d, J = 8.1 Hz, 4H, ArH-Bz). ^{13}C NMR (C_6D_6, 297 K): 14.7 (q), 30.7 (t), 32.0 (q), 35.1 (s), 61.3, 71.7 (t), 127.6, 128.4 (d), 128.9, 129.4 (s), 132.6, 132.8 (d), 134.0 (s), 146.8, 149.3, 149.5 (s), 164.5 (CO), 169.0 (CO).

*5,11,17,23,29,35,41,47-Octa-tert-butyl-56-[carboxy-methoxy]-calix[8]aren-49,50,51,52,53,54,55-hepta-olo (**7**).* Compound **6** (2.90 g, 1.09 mmol) was suspended in EtOH (160 mL) in the presence of 10% aq NaOH solution (30 mL). The mixture was stirred at room temperature for 7 h and then evaporated under vacuum. The solid residue was triturated with 0.1 N HCl and the insoluble material was collected by filtration, washed with water and MeOH, and dried to give white solid **7** (1.40 g, 95%). ^{1}H NMR ($CDCl_3$, 297 K): 1.24 (s, 63H, 7 × *tert*-Bu), 1.25 (s, 9H, *tert*-Bu), 3.91 (s, 16H, 8 × $ArCH_2Ar$), 4.45 (s, 2H, OCH_2), 7.12−7.17 (overlapped, 14H, 14 × ArH), 7.27 (s, 2H, 2 × ArH), 8.86 (s, 2H, 2 × OH), 9.17 (s, 2H, 2 × OH), 9.47 (s, 3H, 3 × OH).

*5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55-hepta-propoxy-56-[propoxy-carbonyl-methoxy]-calix[8]arene (**8**).* Compound **7** (1.40 g, 1.03 mmol) in dry DMF (25 mL) was reacted with *n*-PrI (3.0 mL, 30.8 mmol) in the presence of Cs_2CO_3 (6.7 g, 20.6 mmol) under stirring at room temperature for 24 h. The reaction mixture was concentrated under vacuum and the solid residue was triturated with 0.1 N HCl. The insoluble material was collected by filtration, washed with water and MeOH, dried, and purified by column chromathography (AcOEt/hexane, 2:98 *v*/*v*) to give white solid **8** (1.31 g, 75%). ^{1}H NMR (C_6D_6, 297 K): δ 0.65 (t, J = 6.8 Hz, 3H, CH_2CH_3), 0.75−0.90 (overlapped, 21H, 7 × CH_2CH_3), 1.21 (s, 9H, *tert*-Bu), 1.26 (s, 9H, *tert*-Bu), 1.28 (s, 18H, 2 × *tert*-Bu), 1.29 (s, 27H, 3 × *tert*-Bu), 1.31 (s, 9H, *tert*-Bu), 1.35 (m, 2H, CH_2CH_3), 1.50−1.65 (overlapped, 14H, 7 × CH_2CH_3), 3.51−3.61 (overlapped, 14H, 7 × OCH_2CH_2), 3.92 (t, J = 6.7 Hz, 2H, $COOCH_2CH_2CH_3$), 4.31 (br s, 16H, 8 × $ArCH_2Ar$), 4.40 (s, 2H, OCH_2CO), 7.23 (s, 2H, 2 × ArH), 7.25−7.35 (m, 12H, 12 × ArH), 7.44 (s, 2H, 2 × ArH). ^{13}C NMR ($CDCl_3$, 297 K): δ 11.0, 11.4 (q), 22.9, 24.6, 31.5, 31.6 (t), 32.5 (q), 35.1 (s), 67.0, 71.2, 75.4, 75.7 (t), 126.5, 126.8, 126.9, 127.1, 127.4, 127.8, 128.9 (d), 128.5, 134.1, 134.5, 134.8, 146.5, 146.7, 147.4, 154.8, 154.9, 155.1 (s), 169.0 (CO).

*5,11,17,23,29,35,41,47-Octa-nitro-49,50,51,52,53,54,55-hepta-propoxy-56-[propoxy-carbonyl-methoxy]-calix[8]-arene (**9**).* A solution of **8** (1.30 g, 0.77 mmol) in CH_2Cl_2 (25 mL) was mixed with glacial CH_3COOH (25 mL) at room temperature. After 100% HNO_3 (6 mL) was added dropwise under vigorous stirring. After 6 h, the reaction was stopped by addition of water (120 mL). The organic layer was separated, dried (Na_2SO_4), and the solvent evaporated under vacuum. The yellow solid was purified by column chromathography (gradient 100% CH_2Cl_2 to 1:99 Et_2O/CH_2Cl_2 *v*/*v*) to give white compound **9** (0.50 g, 40%). ^{1}H NMR ($CDCl_3$, 297 K): δ 0.70−1.06 (overlapped, 24H, 8 × CH_2CH_3), 1.60−1.85 (overlapped, 16H, 8 × CH_2CH_3), 3.82 (br s, 14H, 7 × OCH_2), 4.10−4.32 (overlapped, 18H, 8 × $ArCH_2Ar$, $COOCH_2$), 4.58 (s, 2H, OCH_2CO), 7.69−7.87 (overlapped, 16H, 16 × ArH). ^{13}C NMR ($CDCl_3$, 297 K): δ 10.2 (q), 21.9, 23.4, 23.5, 31.0 (t), 124.5, 124.7 (d), 134.2, 134.4, 134.5, 143.9, 161.1 (s), 168.0 (CO). ESI-MS: m/z calcd for $C_{82}H_{90}N_8NaO_{26}$ $[M+Na]^+$ 1625.6; found m/z 1626.6.

*5,11,17,23,29,35,41,47-Octa-amino-49,50,51,52,53,54,55-hepta-propoxy-56-[propoxy-carbonyl-methoxy]calix[8]arene (**10**).* Compound **9** (0.50 g, 0.31 mmol) was dissolved in 200 mL AcOEt/toluene (3:2, *v*/*v*) and hydrogenated under atmospheric pressure in the presence of Pd/C at 50 °C. After the hydrogen uptake was completed (48 h), the catalyst was removed by filtration on Celite and the filtrate was concentrated under vacuum to give pure solid compound **10** (0.38 g, 90%). ^{1}H NMR ($CDCl_3$, 297 K): δ 0.88−1.05 (overlapped, 24H, 8 × CH_2CH_3), 1.73−1.90 (overlapped, 16H, 8 × CH_2CH_3), 3.40−3.76 (overlapped, 14H, 7 × $OCH_2CH_2CH_3$), 3.76−4.05 (overlapped, 16H, 8 × $ArCH_2Ar$), 4.12 (m, 2H, $COOCH_2$), 4.25 (s, 2H, OCH_2CO), 6.10−6.30 (m, 16H, 16 × ArH). ESI-MS: m/z calcd for $C_{82}H_{107}N_8O_{10}$ $[M+H]^+$ 1363.8; found m/z 1364.2.

*5,11,17,23,29,35,41,47-Octa-(Boc-L-glycyl)amino-49,50,51,52,53,54,55-hepta-propoxy-56-[propoxy-carbonyl-methoxy]-calix[8]arene (**11**).* A suspension of Boc-glycine-OH (128 mg, 0.73 mmol), PyBop (570 mg, 1.1 mmol), and DIPEA (0.21 mL, 2.19 mmol) in dry DMF (3 mL) was stirred under argon, at room temperature. After 15 min compound **10** (100 mg, 0.073 mmol) dissolved in dry DMF (4 mL) was added. The reaction mixture was stirred at room temperature overnight. The solvent was removed under vacuum; the crude product was washed with 0.1 N HCl and collected by filtration to give a white solid, which after column chromathography (gradient CH_2Cl_2/MeOH 98:2 to 96:4, *v*/*v*) gave pure compound **11** (140 mg, 73%). ^{1}H NMR ($CDCl_3$/MeOD 4:1, *v*/*v*, 297 K): δ 0.75−0.88 (overlapped, 24H, 8 × CH_2CH_3), 1.41 (s, 72H, 8 × Boc), 1.56 (br m, 16H, 8 × CH_2CH_3), 3.51 (br m, 14H, 7 × $OCH_2CH_2CH_3$), 3.76 (br s, 16H, 8 × gly CH_2), 3.96 (overlapped, 18H, 8 × $ArCH_2Ar$ and $COOCH_2$), 4.29 (s, 2H, OCH_2CO), 7.00−7.20 (m, 16H, 16 × ArH). ^{13}C NMR ($CDCl_3$/MeOD 4:1, 297 K): δ 10.7, 11.1 (q), 22.9, 24.4 (t), 28.8 (q), 31.2, 31.4, 45.0, 67.7, 71.1, 76.4 (t), 80.7 (s), 121.0, 122.3 (d), 135.4, 135.5, 153.4, 158.2 (s), 170.0, 170.8 (CO). ESI-MS: m/z calcd for $C_{138}H_{194}N_{16}Na_2O_{34}$: 1332.7 $[M+2Na]^{2+}$; found m/z 1333.8.

*5,11,17,23,29,35,41,47-Octa-(Boc-L-glycyl)amino-49,50,51,52,53,54,55-hepta-propoxy-56-[carboxy-methoxy]-calix[8]arene (**12**).* Compound **11** (130 mg, 0.049 mmol) suspended in THF (5 mL) in the presence of 2.5% aq. KOH solution (1 mL), was refluxed and stirred at room temperature for 3 h. The reaction was stopped by adding 0.1 N HCl up to pH 4. The product was extracted with CH_2Cl_2, then the organic layer was dried over Na_2SO_4 and evaporated to give pure compound **12** (121 mg, 94%). ^{1}H NMR ($CDCl_3$/MeOD 2:1, 297 K): δ 0.62−0.70 (overlapped, 21H, 7 × CH_2CH_3), 1.21 (s, 72H, 8 × Boc), 1.40−1.48 (m, 14H, 7 × CH_2CH_3), 3.15−4.08 (overlapped, 48H, 8 × gly CH_2, 8 × OCH_2, 8 × $ArCH_2Ar$), 6.50−7.08 (overlapped, 16H, 16 × ArH). ^{13}C NMR ($CDCl_3$/MeOD 4:1, *v*/*v*, 297 K): δ 10.7 (q), 23.8 (t), 28.5 (q), 30.6 (t) 44.5 (t), 75.7 (t), 80.5 (s), 121.8 (d), 134.0, 134.8, 152.7 (s), 157.3 (gly CO) 169.0 (Boc CO). ESI-MS: m/z calcd for $C_{135}H_{188}N_{16}Na_2O_{34}$:1311.7 $[M+2Na]^{2+}$; found m/z 1312.8.

*5,11,17,23,29,35,41,47-tetra-(Boc-L-glycyl)amino-49,50,51,52,53,54,55-hepta-propoxy-56-N-{N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl}-aminoethylamino-carbonyl-methoxy]-calix[8]arene (**13**).*

Compound **12** (100 mg, 0.039 mmol) dissolved in dry DMF (10 mL) was reacted with PyBop (65 mg, 0.125 mmol) and DIPEA (100 μL, 0.57 mmol). After stirring at room temperature for 10 min, *N*-{*N*-palmitoyl-*S*-[2,3-bis-(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl}-1,2-diaminoethane (P_3CS derivative) (55 mg, 0.053 mmol) was added. The reaction mixture was stirred at room temperature overnight. After evaporation of the solvent under vacuum the residue was triturated with 0.1 N HCl (50 mL). The insoluble material was collected by filtration, washed with water, and purified by column chromatography (gradient CH_2Cl_2/MeOH 98:2 to 96:4, *v*/*v*) to give pure compound **13** (117 mg, 83%). ^{1}H NMR ($CDCl_3$/MeOD 1:1 *v*/*v*, 297 K): δ 0.80 (br s, 21H, 7 × CH_2CH_3), 0.84 (t, *J* = 6.4 Hz, 9H, 3 × palmitoyl CH_3), 1.22 (br s, 72H, 36 × palmitoyl CH_2), 1.39 (br s, 72H, 8 × Boc), 1.57 (br m, 20H, 7 × CH_2CH_3 and 3 × palmitoyl CH_2CH_3), 2.12−2.30 (m, 6H, 3 × palmytoyl CH_2CO), 2.75 (br m, 2H, SCH_2CHOCO), 2.84 and 3.02 (m, 1H each, cysβ-CH_2), 3.50 (br m, 14H, 7 × $OCH_2CH_2CH_3$), 3.60−4.01 (overlapped 40H, 8 × $ArCH_2Ar$, 8 × gly CH_2, OCH_2CO, NCH_2CH_2N, Ser $CHCH_2OH$), 4.03−4.35 (overlapped 3H, Ser $CHCH_2OH$, SCH_2CHCH_2), 4.58 (overlapped H_2O, 1H, cysα-CH), 5.09 (m, 1H, SCH_2CHOCO), 6.80−7.23 (overlapped, 16H, 16 × ArH). ^{13}C NMR ($CDCl_3$/MeOD 1:1 *v*/*v*, 297 K): δ 10.7, 14.3 (q), 23.2, 23.9, 25.4, 26.3 (t), 28.6 (q), 29.7, 29.9, 30.2, 32.4, 34.5, 34.6, 34.8, 36.6, 37.9, 38.0, 44.5 (t), 53.8, 57.0 (d), 60.4, 62.2, (t), 68.7 (d), 75.7 (t), 80.5 (s), 121.7, 121.8, 121.9, 122.0 (d), 132.4, 133.9, 134.3, 134.9, 152.5, 152.7, 152.9, 157.4, 169.1, 174.2, 174.5.

*5,11,17,23,29,35,41,47-tetra-(L-glycyl)amino-49,50,51,52,53,54,55-hepta-propoxy-56-N-{N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl}-aminoethylamino-carbonyl-methoxy]-calix[8]arene (**13a**).* Compound **13** (117 mg, 0.032 mmol) was stirred with TFA (0.6 mL) at room temperature for 2 h. The mixture was dried under vacuum, dissolved in ethanol, and evaporated (three times). After treatment with 0.1 N HCl, pure compound **13a** (100 mg) in Cl^- saline form was obtained in quantitative yield. The removal of the Boc groups was confirmed by the disappearance of the resonances at 1.39 ppm in the proton spectrum of **13a** and by the presence of two significant peaks in MALDI-TOF spectrum (reflector mode): *m*/*z* calcd for $C_{154}H_{237}N_{20}O_{25}S$: 2800.7 $[M+H]^+$; found *m*/*z* 2799.3; *m*/*z* calcd for $C_{154}H_{236}N_{20}NaO_{25}S$: 2822.7 $[M+Na]^+$; found *m*/*z* 2821.3.

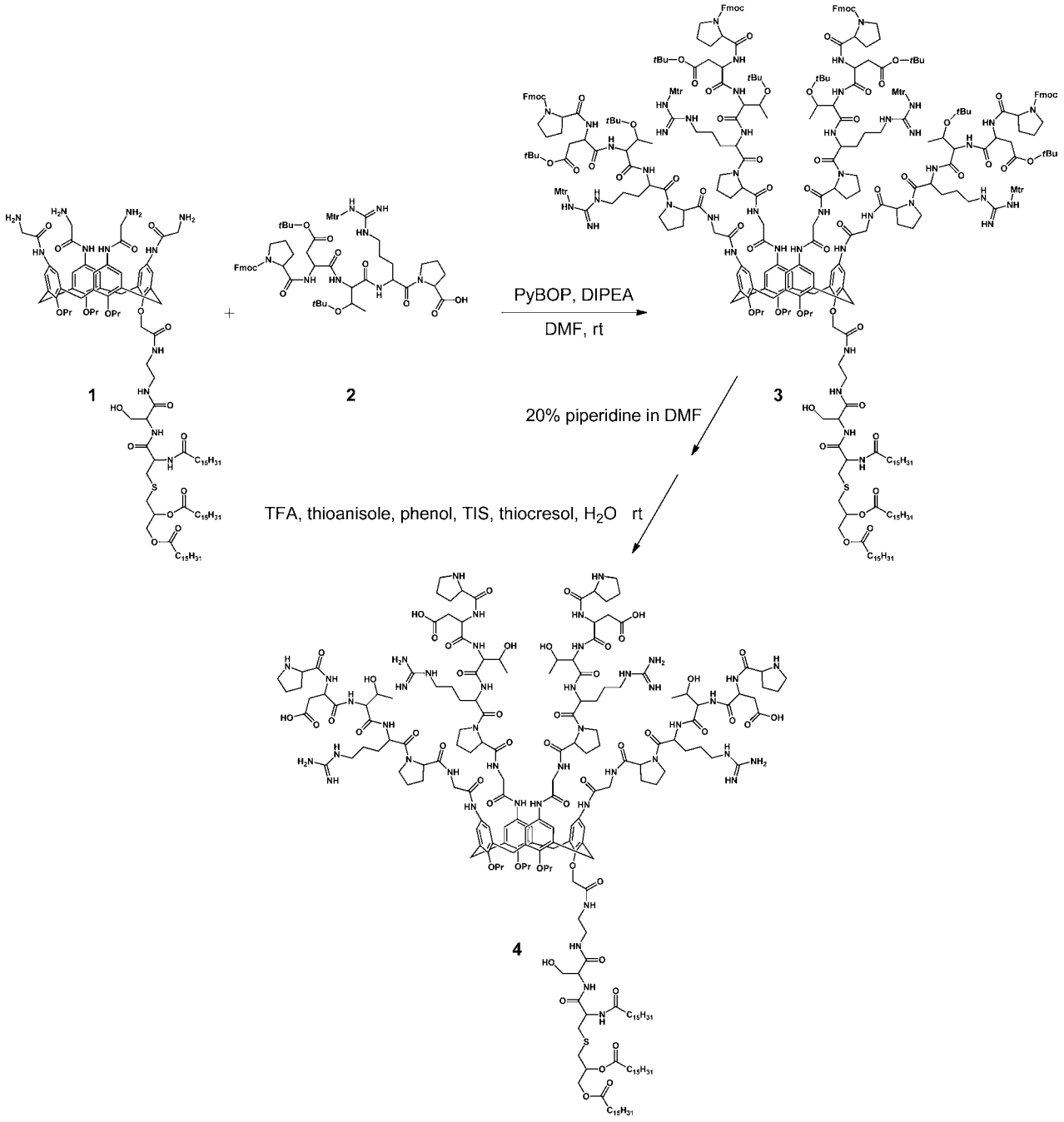
*Octa-protected PDTRP-Gly-C8-P_3CS (**14**).* To PDTRP peptide **2** (170 mg, 0.15 mmol) dissolved in dry DMF (10 mL), PyBop (119 mg, 0.23 mmol) and DIPEA (100 μL, 0.57 mmol) were added. The mixture was stirred at room temperature under argon for 10 min, then calixarene derivative **13a** (49 mg, 0.016 mmol) was added. The stirring was continued at room temperature overnight. Then, the solvent was removed under vacuum and the solid triturated with 0.1 N HCl was collected by filtration. Pure compound **14** (79 mg) was obtained in 42% yield after gel permeation chromatography (Sephadex LH-20, CH_2Cl_2 as eluent) and preparative TLC (CH_2Cl_2/MeOH 1:9, *v*/*v*). Because of the overlap of many signals in the ^{1}H NMR spectrum, we report the chemical shift values of some diagnostic signals. ^{1}H NMR ($CDCl_3$/MeOD 4:1 *v*/*v*, 297 K): δ 0.79 (br t, 9H, 3 × palmitoyl CH_3), 0.81−1.01 (overlapped, 45 H, 7 × propyl CH_3 and 8 × thr CH_3), 1.06 (br s, 72H, 8 × thr *tert*-Bu), 1.17 (br s, 72H, 36 × palmitoyl CH_2), 1.29 (s, 72H, 8 × aspartate *tert*-Bu), 1.50 (br m, 9H, 3 × palmitoyl CH_2CH_3), 6.40 (br s, 8H, 8 × calixarene ArH), 6.43 (s, 8H, 8 × Mtr CH), 7.15 (br s, 8H, 8 × calixarene ArH), 7.21 (br m, 16H, 16 × Fmoc ArH), 7.31 (br m, 16H, 16 × Fmoc ArH), 7.49 (br d, 16H, 16 × Fmoc ArH), 7.67 (br d, 16H, 16 × Fmoc ArH). ^{13}C NMR ($CDCl_3$/MeOD 4:1 *v*/*v*, 297 K): 10.0, 11.5,13.6, 17.9 (q), 22.3, 23.1 (t), 23.6, 23.9(q), 24.2, 24.6 (t), 27.4, 27.7 (q), 28.8, 29.0, 29.3, 30.9, 31.2, 1, 31.6, 33.8, 34.0, 35.8, 36.3, 36.9, 39.9, 42.8, 46.8, 47.2 (t), 46.9, 48.7, 49.3, 49.6, 50.4, 55.0, 57.7, 60.4, 66.1, 70.0 (d), 67.5 (t), 74.7, 81.5 (s), 111.4 (d), 119.7, 121.0, (d), 124.4 (s), 124.7, 126.8, 127.5 (d), 133.2, 133.9, 136.3, 138.1, 141.0, 143.4, 143.6, 143.9, 152.0, 155.0, 155.5, 156.2, 158.2, 167.5, 169.1, 170.4, 172.7 (s).

*Octa-PDTRP-Gly-C8-P_3CS (**15**).* Compound **14** (75 mg, 64 μmol) was stirred with piperidine (20% DMF solution, 0.96 mL) at room temperature for 2 h. The solvent was removed under vacuum and the crude product was purified on Sephadex LH-20 (CH_2Cl_2 as eluent) to give the corresponding de-Fmoc derivative (58 mg) in 91% yield. The removal of the Fmoc groups was confirmed by the disappearance of the relative resonances in the ^{1}H NMR spectrum. To remove the remaining protective groups, the compound (50 mg, 50 μmol) was treated with 3.2 mL of a mixture of TFA:thioanisole:phenol:TIS:thiocresol:H_2O (8:0.5:0.3:0.25:0.1, *v*/*v*). The mixture was stirred at room temperature for 24 h. The solvent was removed under vacuum, and then the residue was dissolved more times in Et_2O and EtOH and evaporated. Crude compound was purified by size-exclusion chromatography on Sephadex LH-20 (1% MeOH/CH_2Cl_2, *v*/*v*, as eluent). The first fractions were collected and further purified by C18-RP column chromatography (MeOH) to give the pure compound **15** (30 mg, 82%) as a white powder. Because of the overlap of many signals in the ^{1}H NMR spectrum, we report the chemical shift values of some diagnostic signals. ^{1}H NMR (MeOD, 330 K): δ 0.86 (br t, 9H, 3 × palmitoyl CH_3), 1.15 (br s, 45H, 7 × propyl CH_3 and 8 × thr CH_3), 1.27 (br s, 72H, 36 × palmitoyl CH_2), 4.75 (br s, 8H, 8 × thr CH) 7.20 (br s, 16H, 16 × calixarene ArH). MALDI-TOF *m*/*z* calcd for $C_{346}H_{541}N_{84}O_{89}S$ 7333.6 $[M+H]^+$; found *m*/*z* 7340.2; *m*/*z* calcd for $C_{346}H_{539}KN_{84}NaO_{89}S$ 7393.6 $[M+Na+K-H]^+$; found *m*/*z* 7397.2.

Animal Treatment. Five- to eight week-old female BALB/c mice (Charles River, Calco, Italy) were used in this study. Five groups of six mice were immunized two times at 1-week intervals by intraperitoneally injections of **15** (0.030 μM/mouse), **4** (0.030 μM/mouse), and **16**[15] at three concentration levels (0.030, 0.120, and 0.240 μm/mouse). Two additional groups were injected with **15** and **16** adducts deprived of PDTRP peptide moieties. All the constructs administered were dispersed in 500 μL of a saline solution containing 20 mg/mL of β-HP-CD and 5 mg/mL of HPMC. These additives enhance solubility and physical stability of the dispersion of the constructs tested. Finally, two other groups were injected with vehicle alone or not treated. After 21 days from the first immunization, sera were collected and stored at −80 °C until ELISA assay.

ELISA End-Point Titers. Total IgG antibodies specific to PDTRP antigen in sera were detected as previously reported[16] and quantified by end-point dilution ELISA assay on samples pooled for each group. End-point titers were defined as the highest serum dilution that resulted in an absorbance value two times greater than that of non-immune control mouse serum with a cutoff value of 0.05.[7,17] Briefly, ELISA 96-wells plate (Corning 9018 or Nunc Maxisorp) were coated overnight at 4 °C with PDTRPDTRPDTRP peptide (5 mg/mL) and

Scheme 1. Synthesis of Tetra-PDTRP-Gly-C4-P_3CS (4)



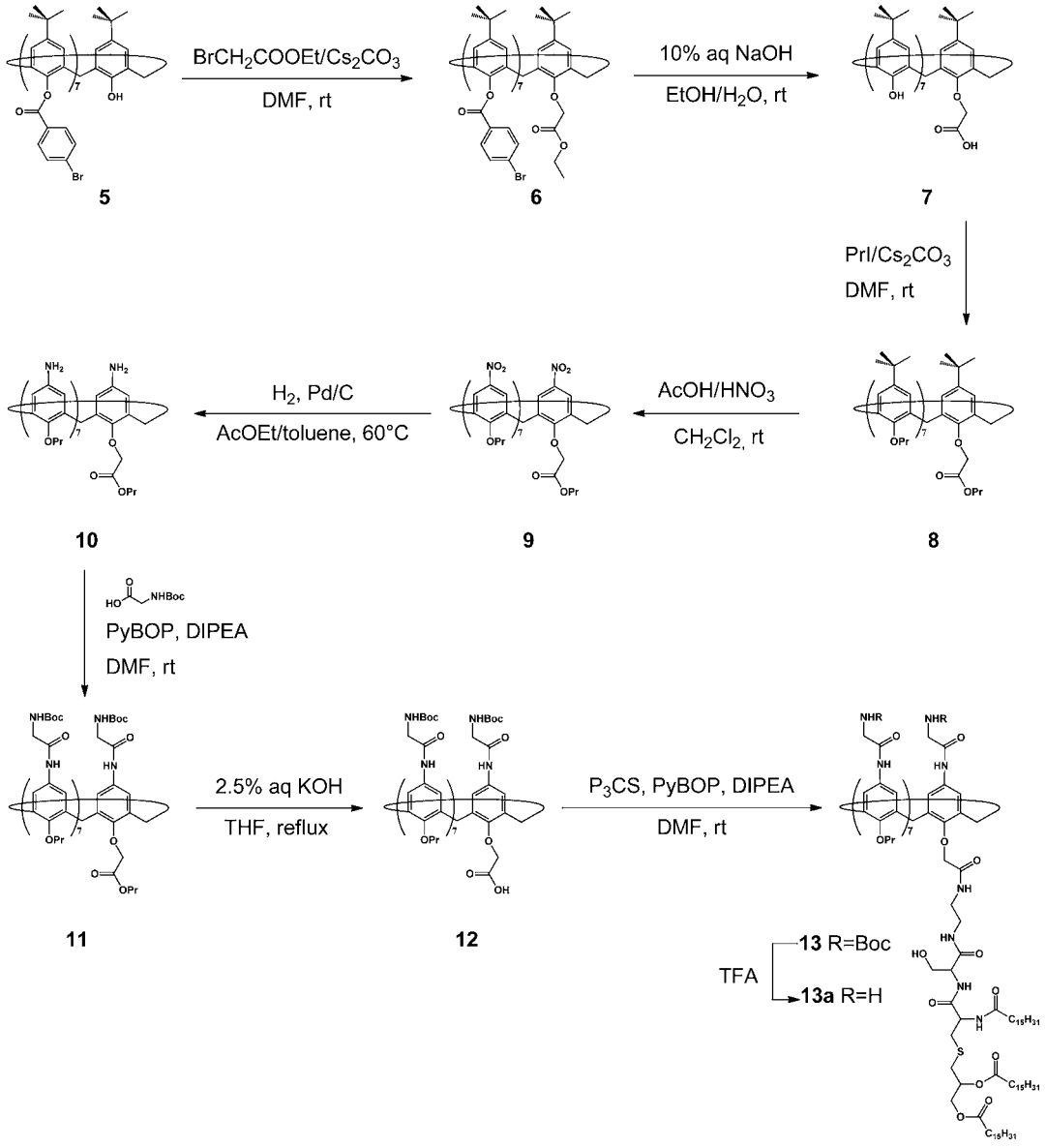
incubated with individual mouse sera at different dilutions. After incubation with horseradish peroxidase-conjugated goat antimouse IgG (Sigma), color was developed using phenylenediamine dihydrochloride and H_2O_2 as substrate and absorbance at 450 nm was measured in an Elisa microplate reader.

Cell Cultures. Human breast adenocarcinoma cells MCF7,[18,19] obtained from ATCC, were cultured (37 °C and 5% CO_2) in Eagle's minimum essential medium with L-glutamine (2 mM) and Earle's balanced salt solution adjusted to contain sodium bicarbonate (1.5 g/L), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), bovine insulin (0.01 mg/mL) and supplemented with fetal bovine serum (10%).

MCF7 Cell Recognition Evaluation. The capacity of the mouse sera to recognize native MUC1 antigen present on cancer cells was established as previously reported[20] on MUC1-expressing MCF7. Briefly, serial dilutions of pre- and postimmunization sera were incubated with MCF7 single-cell suspensions. Successively, the cells were washed and incubated with goat antibodies to IgG (gamma chain specific) conjugated to fluorescein isothiocyanate (Sigma). Finally the cells were lysed and the lysates were analyzed for fluorescence intensity (λ_{ex} = 485/λ_{em} = 520) with a microplate reader. Data points were collected in triplicate, and are representative of five independent experiments.

Statistical Analysis. Data from ELISA and cell recognition analysis were analyzed by Two Sample Ranks Test to

Scheme 2. Synthesis of Compound 13a



determine statistical significance and to estimate the p value when comparing different groups within an experiment ($p < 0.05$ was considered significant).

RESULTS AND DISCUSSION

Design of the Synthetic Vaccine Candidate. In a previous work, in order to mimic the clustered motif of Tn antigen recognized by the immune system, we have synthesized one self-adjuvant tetravalent vaccine candidate by clustering glycomimetic of Tn antigens and incorporating TLR2 ligand on a "nonpeptidic" calix[4]arene platform. This construct, with a well defined spatial preorganization of the antigenic moieties, elicited a higher specific imumostimulating activity in mice when compared to its monovalent analogous compound, and the outlined data showed a multivalency effect.[7]

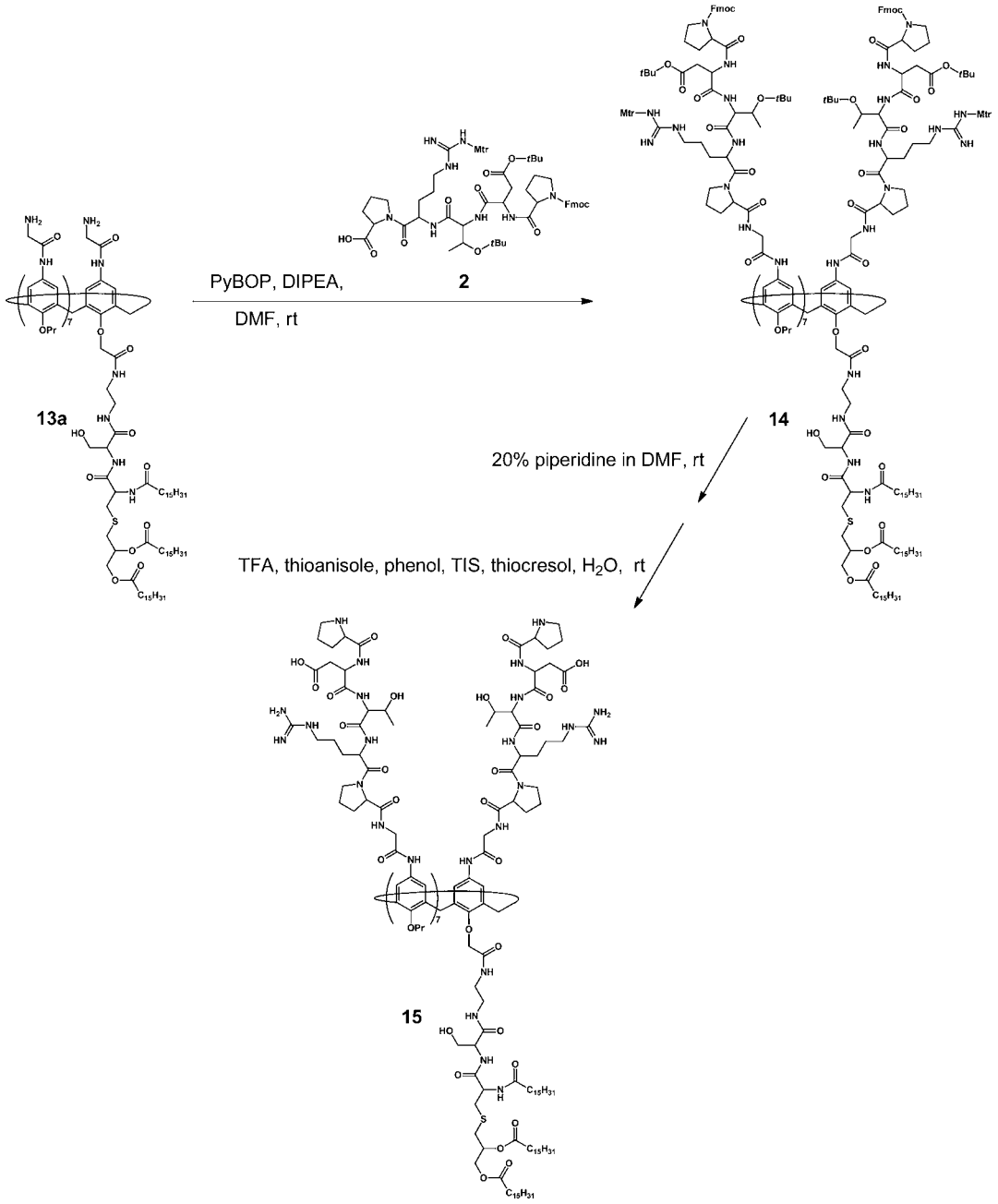
In a program aimed at the development of MUC1 anticancer vaccines versus epithelial tumors such as breast, pancreatic, lung, colorectal, and ovarian cancer, we have planned to exploit the immunodominant PDTRP motif to develop multivalent PDTRP-based vaccine candidates able to elicit a specific immune response. Different clusters of tumor-associated carbohydrate antigens were described,[7,21] but no data focused on the clusterization of PDTRP peptide MUC1 fragment. The designed constructs consist of clusters of PDTRP antigen and one immunoadjuvant P_3CS group assembled on calixarene scaffolds.

As it is known, the calixarene macrocycles are suitable platforms for application in molecular recognition phenomena[22,23] and for studying the multivalency effect.[24,25] They allow one to selectively vary the number and the type of ligands introduced on the platform, and for this reason they have found application in different biomedical field[26] as enzyme inhibitors,[27−30] anticoagulant and antithrombotic,[31] antiangiogenic and anticancer,[32−34] antiviral,[35,36] antibacterial, and antifungal products.[37−39]

In the present work, we have selected the smaller calix[4]arene tetramer and the larger homologous calix[8]arene as macrocycle skeletons for the conjugation of four or eight PDTRP immunodominant units, respectively, in order to investigate the multivalency effect in amplifying the specific

dx.doi.org/10.1021/bc400242y | Bioconjugate Chem. 2013, 24, 1710−1720

Scheme 3. Synthesis of Octa-PDTRP-Gly-C8-P_3CS (15)



antibody production. In the synthesized constructs, the PDTRP units are covalently linked via an amide bond to the calixarene wide rim, whereas a P_3CS unit and propyloxy groups are present at the narrow rim of the calixarene macrocycle.

In addition, the rigid calix[4]arene and the more flexible calix[8]arene skeleton also allow one to investigate how tridimensional architecture and conformational mobility can affect the biological activity. The rigid calixarene platform, consisting of a calix[4]arene scaffold fixed in a cone conformation by the presence at the narrow rim of three propyloxy groups and one P_3CS, enables one to arrange four PDTRP epitopes in all-syn orientation onto the calixarene platform; instead, the flexible calix[8]arene skeleton, in a mobile pleated-loop conformation, enables one to arrange eight PDTRP functions in a different spatial orientation. The conformational mobility could be advantageous in improving avidity and selectivity of the ligand−receptor interaction and in recognition events favoring molecular adaptive interactions.[40−42] Recent development in drug design has considered mutual adaptability between a ligand and a receptor as a key element in molecular recognition.[43]

In the synthesized constructs, the P_3CS lipopeptide moiety determines the magnitude of the vaccine candidate immune response. In fact, it is a potent immunoadjuvant which interacts with TLR2.[10,13] The novel fully synthetic molecularly and chemically well-defined constructs contain all the elements required for relevant and reproducible immune responses, and they do not need any external adjuvant or foreign conjugate carrier proteins. These latter, present in numerous anticancer vaccines, are highly immunogenic and could lead to the suppression of specific antibody response.[44]

dx.doi.org/10.1021/bc400242y | Bioconjugate Chem. 2013, 24, 1710−1720

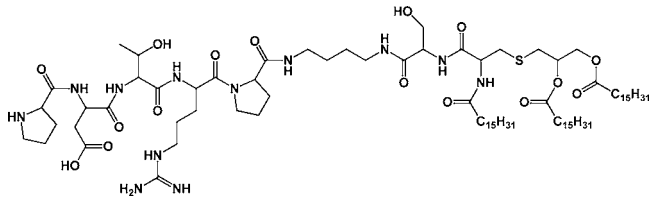
Synthesis of the Conformationally Blocked Calixarene-Based Vaccine. To conjugate four units of the pentapeptide PDTRP onto the calix[4]arene platform (Scheme 1), the amino derivative **1** was reacted with the protected PDTRP sequence (**2**) through the free carboxyl of the terminal prolin, in the presence of PyBOP and DIPEA in DMF at room temperature, to give derivative **3** in 60% yield. The ^{1}H NMR spectrum of compound **3**, though very complex for overlap of many signals, showed the characteristic resonances for P_3CS group (δ 0.79, 3 × palmitoyl-CH_3), calixarene $ArCH_2Ar$ (δ 2.98 and 4.25, 3.02 and 4.27), and protecting groups of PDTRP (4 × Fmoc, 4 × Mtr, and 8 × *tert*-Bu), thus confirming the wide rim exhaustive functionalization. The Fmoc groups of compound **3** were removed with 20% piperidine solution in DMF and subsequently the remaining protective groups were taken away, by treatment with a mixture of trifluoroacetic acid, thioanisol, phenol, triisopropylsilane, thiocresol, and H_2O for two days at room temperature, to give compound **4** in 53% yield. The ^{1}H NMR spectrum of **4** presented overlapping resonances which made the signal assignment very difficult, but the disappearance of all proton signals relative to the protecting groups corroborated the deprotection. The structure of final construct **4** was also confirmed by MALDI-TOF mass spectrum, which showed indicative signals at *m/z* 4188, 4205, and 4221 corresponding to $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions, respectively.

Synthesis of the Conformationally Flexible Calixarene-Based Vaccine. To introduce one P_3CS group at the lower rim of the calix[8]arene (Scheme 2), we synthesized the hepta-bromobenzoyloxy derivative (**5**), according to a previously reported procedure.[13] This compound was reacted with $BrCH_2COOEt$ in the presence of Cs_2CO_3, as a base, to obtain compound **6** in high yield (90%).

In the ^{1}H NMR spectrum, diagnostic signals at δ 0.74, 3.73, and 4.11 relative to the OCH_2COOEt substituent confirmed the alkylation of the free OH group. Hydrolysis of **6** and subsequent alkylation with *n*-PrI in the presense of Cs_2CO_3 provided compound **8** substituted at the lower rim by seven propoxy groups and one aryl-methoxycarbonyl-propoxy group as confirmed by ^{1}H NMR spectrum (δ 0.75−0.90 for $OCH_2CH_2CH_3$ and δ 0.65 for $OCOCH_2CH_2CH_3$). This compound underwent a direct replacement of the *tert*-butyl groups via an *ipso*-nitration to give the nitro-calixarene derivative (**9**). The ^{1}H NMR spectrum by the disappearance of the *tert*-butyl groups signals and downfield shift of the ArH signals (δ 7.69−7.87). Subsequently, reduction of **9** by catalytic (Pd/C) hydrogenation in H_2 atmospheric pressure supplied the amino derivative (**10**) which showed typical upfield shift of the ArH signals (δ 6.10−6.40) in the protonic spectrum. Afterward, eight Boc-glycine spacers were introduced into macrocycle wide rim, via amide coupling, in the presence of PyBOP and DIPEA to give compound **11**. The exhaustive functionalization of the upper rim was confirmed by the signal at δ 1.21 (8 × Boc) and δ 3.76 (8 × gly CH_2) in the ^{1}H NMR spectrum. To allow the conjugation of the P_3CS imunoadjuvant group at the calixarene lower rim, compound **11** was reacted with a 2.5% aq. KOH solution to give compound **12** bearing a terminal COOH group. Compound **12** was reacted with P_3CS in the presence of PyBOP and DIPEA to provide compound **13** in 84% yield. In the proton spectrum of **13**, the presence of signals corresponding to the protons of the palmitoyl, cysteinyl, and seryl groups of the P_3CS confirmed the introduction of the immuonoadjuvant. Compound **13** after removal of the Boc groups with TFA and replacing of CF_3COO^- counterions with Cl^- anions (compound **13a**) was reacted with protected PDTRP peptide (**2**) using PyBOP and DIPEA (Scheme 3) to obtain **14**. Despite the overlap of many resonances in the ^{1}H NMR spectrum, some signals were diagnostic to demonstrate the structure of compound **14**. Furthemore, the exhaustive functionalization of the macrocyle wide rim was confirmed by the integration of the proton signals corresponding to eight Fmoc, eight Mtr, and sixteen *tert*-Bu groups.

Finally, the removal of all protecting groups of compound **14** by means of piperidine and subsequent treatment with a mixture of TFA, thioanisole, phenol, TIS, thiocresol, and H_2O gave the final construct **15** in 75% yield. The very complex ^{1}H NMR spectrum showed the absence of all the protecting groups and in MALDI-TOF spectrum peaks at *m/z* 7340 $[M+H]^+$ and 7397 $[M+Na+K-H]^+$ were diagnostic to confirm the structure of **15**.

Chart 1. Structure of Monovalent Reference Compound (16)



Immunological Evaluation. The immunogenicity of the multiepitopic constructs (**4** and **15**) was evaluated in wild Balb/c mice by ELISA test, and compared to that of a reference monoepitopic[15] construct (**16**), in which a single antigenic sequence PDTRP was conjugated through a spacer to the immunoadjuvante P_3CS (Chart 1). Data reported in Table 1 show that mice treated with equimolar amount (0.030 μM/mouse) of construct **4**, **15**, or **16** exhibited a higher total PDTRP specific IgG antibody production than the control animals, with an increase order of **15** > **4** > **16**. In particular, in the serum dilution range examined, mice immunized with **15** and **4** showed end-point titers 32- and 8-fold higher than mice groups immunized with the reference monovalent construct. Moreover, octamer **15** revealed the most active compound with a significantly 4-fold higher ($p < 0.05$), instead of 2-fold, antibody production than tetramer **4**.

Table 1. Reciprocal Median Peak ELISA Titers after Immunization of Groups of Six Mice with the Multivalent Antigenic Constructs 4, 15, and the Monovalent Construct 16

Construct	IgG Anti-PDTRP
15	5120
4	1280
16 (8×)	640[a]
16 (4×)	320[a]
16 (1×)	160

[a] **16** (8×) vs **16** (4×) $p = 0.132$ (two samples rank test). All other comparisons were significant ($p < 0.05$).

In order to evaluate whether the higher antibody production observed for constructs **4** and **15** was due to the presence of a major number of epitope units, the reference monovalent

dx.doi.org/10.1021/bc400242y | *Bioconjugate Chem.* 2013, 24, 1710−1720

construct (**16**) was administrated at molar concentration 4× (0.120 μM/mouse) and 8× (0.240 μM/mouse) with respect to **4** and **15**. The obtained results permitted us to rule out an epitope concentration effect because they showed that **16** in concentrations 4× and 8× continued to stimulate lower antibody production than **4** and **15** (see Table 1). The 4-fold and 8-fold higher IgG production observed for **4** and **15** also evidenced that epitope units clustered by the calixarene scaffolds elicit a more effective immunostimulating activity than the same number of free epitope units. Taken together, these data clearly show that multivalent binding interactions[45,46] are operative for both multivalent constructs, and the effect is more pronounced in the octameric derivative.

The conformational flexibility of the calix[8]arene scaffold could provide a better spatial arrangement of the PDTRP antigens, which ensures effective molecular recognition interaction with the MHC antigen processing machinery. In fact, it is known that the calix[8]arene derivatives with nonrestricted conformational flexibility are suitable to ligand–receptor recognition by mutually induced fit process.[42]

Interestingly, when the concentration of the construct **16** was increased from 1× to 4× and 8×, the end-point titers increased only by 2- and 4-fold rather than 4- and 8-fold. The small and not statistically significant (p = 0.132) antibody response suggests that the mouse immunological system is not responsive to a concentration increment and tends to saturate.

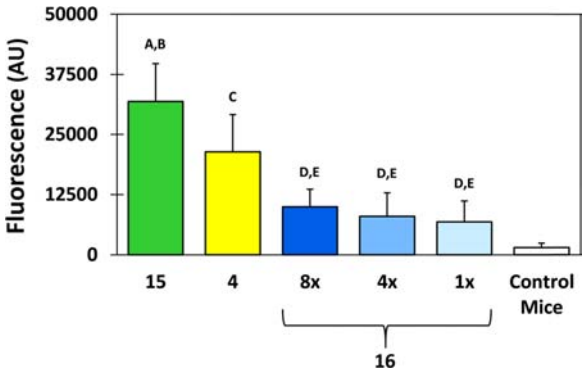


Figure 1. Cell recognition analysis for specific anti-MUC1 antibodies tested on MCF7 cells. Sera of mice were evaluated after immunizations with **15**, **4**, and the reference nonclustered construct **16** (administered at three concentration levels: 1×, 4×, and 8× ratio molar excess). Data points represent the mean value from group of five animals. [A] $p < 0.05$ compared with **16** (8×, 4×, and 1×) and control; [B] $p > 0.05$ compared with **4**; [C] $p < 0.05$ compared with **16** (8×, 4×, and 1×) and control; [D] $p > 0.05$ when 8×, 4×, and 1× groups were compared each other; [E] $p < 0.05$ compared with control.

The mice groups treated with calixarene derivatives **1** and **13a** lacking in PDTRP epitopes showed no significant specific antibody production when compared to the control group (data not shown). This result indicates that the calixarene platforms are convenient nonimmunogenic carrier for vaccine constructs.

Finally, to investigate whether the mouse sera from constructs **4**, **15**, and **16** could recognize the native MUC1 epitopes expressed on cancer cells, we analyzed the binding to MUC1-expressing MCF7 human breast cancer cells by use of a FITC-labeled antimouse IgG secondary antibody.[20] As shown in Figure 1, sera antibodies from all three constructs were able to bind MUC1 epitopes on the surface of MCF7 cells. Importantly, antisera obtained from immunizations with the clustered constructs **15** and **4** revealed good and statistically significant ($p < 0.05$) recognition of MUC1 tumor cells, when compared to the nonclustered reference construct **16** (tested at 1×, 4×, and 8× ratio molar excess). The MCF7 recognition by the monovalent construct **16** showed the same trend observed in the ELISA studies. In fact, there is no significant increase in the MUC1 recognition when the concentration of the nonclustered construct **16** were raised from 1× to 8× molar excess. No binding was observed when cells that do not express the MUC1 antigen (SK-MEL-28) were used (data not shown).

CONCLUSIONS

To the best of our knowledge, the constructs synthesized represent the first examples of self-adjuvant vaccine candidates based on a calix[4 or 8]arene platform exposing MUC1 PDTRP immunodominant peptide sequence. The novel constructs, fully synthetic and molecularly well-defined, contain the elements essential for inducing a specific immune response, without conjugation to highly immunogenic carrier proteins.

The arrangement of multiple PDTRP epitopes on a calixarene platform resulted in an increase of the immunological response with respect to the monovalent epitope, and derivative **15** exposing eight PDTRP units was not 2-fold but 4-fold more active than derivative **4** presenting four epitopic units. These data taken together ruled out an additive effect and suggested that a multivalency effect occurs. The major activity of derivative **15** built on the flexible calix[8]arene skeleton could also be explained considering the involvement of supramolecular adaptative phenomena which make more effective the ligand–receptor molecular recognition. As expected, no significant specific antibody production was observed in mice treated with calixarene derivatives **1** and **13a**, lacking in PDTRP peptide moieties. This suggests the potential use of the calixarene platforms as convenient carriers for building new vaccines. Cell recognition analysis on MCF7 tumor cells clearly showed that antibodies induced by constructs **4**, **15**, and **16** efficiently target MUC1-expressing tumor cells.

Encouraged by the results obtained and motivated by recent papers which report an effective immunogenicity of glycosylated PDTRP epitopes,[47] we have already undertaken studies to realize new calixarene-based constructs bearing PDTRP units glycosylated with tumor-associated carbohydrate Tn antigen.

In conclusion, the self-adjuvant multicomponent antitumor calixarene-based vaccine candidates, which contain four or eight PDTRP moieties and a TLR2 ligand (P_3CS), are promising immunotherapeutic antitumor agents that should deserve further study.

AUTHOR INFORMATION

Corresponding Authors

*Corrada Geraci: phone +39 095 7338318; fax +39 095 7338310; e-mail corrada.geraci@icb.cnr.it.

*Angelo Spadaro: phone +39 095 7384011; fax +39 095 7384250; e-mail angelo.spadaro@unict.it.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Finn, O. J. (2008) Cancer Immunology. *N. Engl. J. Med. 358*, 2704–2715.

(2) Richardson, J. P., and Macmillan, D. (2008) Mucin-Based Vaccines. In *Glycoscience* (Fraiser-Reid, B., Tatsuta, K., and Thiem, J., Eds.) pp 2645−2698, Chapter 12.8, Springer-Verlag, Berlin.
(3) Miles, D. W., and Taylor-Papadimitriou, J. (1999) Therapeutic aspects of polymorphic epithelial mucin in adenocarcinoma. *Pharmacol. Ther. 82*, 97−106.
(4) Quinlin, I. S., Burnside, J. S., Dombrowski, K. E., Phillips, C. A., Dolby, N., and Wright, S. E. (2007) Context of MUC1 epitope: Immunogenicity. *Oncol. Rep. 17*, 453−456.
(5) Becker, T., Dziadek, S., Wittrock, S., and Kunz, H. (2006) Synthetic glycopeptides from the mucin family as potential tools in cancer immunotherapy. *Curr. Cancer Drug Targets 6*, 491−517.
(6) Grinstead, J. S., Schuman, J. T, and Campbell, A. P. (2003) Epitope mapping of antigenic MUC1 peptides to breast cancer antibody fragment B27.29: A heteronuclear NMR study. *Biochemistry 42*, 14293−14305.
(7) Geraci, C., Consoli, G. M. L., Galante, E., Bousquet, E., Pappalardo, M., and Spadaro, A. (2008) Calix[4]arene decorated with four Tn antigen glycomimetic units and P3CS immunoadjuvant: synthesis, characterization, and anticancer immunological evaluation. *Bioconjugate Chem. 19*, 751−758.
(8) Bousquet, E., Spadaro, A., Pappalardo, M. S., Bernardini, R., Romeo, R., Panza, L., and Ronsisvalle, G. (2000) Synthesis and immunostimulating activity of a thioglycopeptide glycomimetic as a potential anticancer vaccine derived from Tn antigens. *J. Carbohydr. Chem. 19*, 527−541.
(9) Fasting, C., Schalley, C. A., Weber, M., Seitz, O., Hecht, S., Koksch, B., Dernedde, J., Graf, C., Knapp, E.-W., and Haag, R. (2012) Multivalency as a chemical organization and action principle. *Angew. Chem., Int. Ed. 51*, 10472−10498.
(10) Spohn, R., Buwitt-Beckmann, U., Brock, R., Jung, G., Ulmer, A. J., and Wiesmüller, K. H. (2004) Synthetic lipopeptide adjuvants and Toll-like receptor 2 - Structure-activity relationships. *Vaccine 22*, 2494−2499.
(11) Prass, W., Ringsdorf, H., Reitermann, A., Bessler, W. G., Wiesmüller, K.-H., and Jung, G. (1987) Lipopeptides of the N-terminus of *Escherichia coli* lipoprotein: synthesis, mitogenicity and properties in monolayer experiments. *Biochim. Biophys. Acta 900*, 116−128.
(12) Reichel, F., Roelofsen, A. M., Geurts, H. P. M., Hämäläinen, T. I., Feiters, M. C., and Boons, G.-J. (1999) Stereochemical dependence of the self-assembly of the immunoadjuvants Pam3Cys and Pam3Cys-Ser. *J. Am. Chem. Soc. 121*, 7989−7997.
(13) Aliprantis, A. O., Yang, R.-B., Mark, M. R., Sugett, S., Devaux, B., Radolf, J. D., Klimpel, G, R., Godowsky, P., and Zychlinsky, A. (1999) Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science 285*, 736−739.
(14) Consoli, G. M. L., Cunsolo, F., and Neri, P. (1996) Study on the Esterification of *p-tert*-Butylcalix[8]arene. *J. Org. Chem. 61*, 2195−2198.
(15) Unpublished results.
(16) Chu, Y., Xia, M., Lin, Y., Li, A., Wang, Y., Liu, R., and Xiong, S. (2006) Th2-dominated antitumor immunity induced by DNA immunization with the genes coding for a basal core peptide PDTRP and GM-CSF. *Cancer Gene Ther. 13*, 510−519.
(17) Davis, H. L., Mancini, M., Michel, M. L., and Whalen, R. G. (1996) DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. *Vaccine 14*, 910−915.
(18) Chalmers, J. J., Xiong, Y., Jin, X., Shao, Tong, M. X., Farag, S., and Zborowski, M. (2010) Quantification of non-specific binding of magnetic micro- and nanoparticles using cell tracking velocimetry: implication for magnetic cell separation and detection. *Biotechnol. Bioeng. 105*, 1078−1093.
(19) Horwitz, K. B., Costlow, M. E., and McGuire, W. L. (1975) MCF-7: a human breast cancer cell line with estrogen, androgen, progesterone and glucorticoid receptors. *Steroids 26*, 785−795.
(20) Ingale, S., Wolfert, M. A., Buskas, T., and Boons, G.-J. (2009) Increasing the antigenicity of synthetic tumor-associated carbohydrate antigens by targeting Toll-Like receptors. *ChemBioChem 10*, 455−463.
(21) Fiore, M., Thomas, B., Duléry, V., Dumy, P., and Renaudet, O. (2013) Synthesis of multi-antigenic platforms as vaccine candidates against cancers. *New J. Chem. 37*, 286−289.
(22) Baldini, L., Sansone, F., Casnati, A., and Ungaro, R. (2012) Calixarenes in molecular recognition. *Supramolecular Chemistry: From Molecules to Nanomaterials Vol. 3: Molecular Recognition* (Steed, J. W., and Gale, P. A., Eds.) pp 863−894, John Wiley & Sons.
(23) Perret, F., Peron, H., Dupin, M., and Coleman, A. W. (2007) Calixarenes as Protein Sensors, *Topics in Current Chemistry, Creative Chemical Sensor Systems Vol. 277* (Schrader, T., Ed.) pp 31−88, Springer, Berlin.
(24) Mammen, M., Choi, S.-K., and Whitesides, G. (1998) Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibithors. *Angew. Chem., Int. Ed. 37*, 2754−2794.
(25) Choi, S.-K. (2004) *Synthetic Multivalent molecules: concepts and Biomedical applications*, John Wiley & Sons Inc. Eds., New York.
(26) Nimse, S., and Kim, T. (2013) Biological applications of functionalized calixarenes. *Chem. Soc. Rev. 42*, 366−386.
(27) Francese, S., Cozzolino, A., Caputo, I., Esposito, C., Martino, M., Gaeta, C., Troisi, F., and Neri, P. (2005) Transglutaminase surface recognition by peptidocalix[4]arene diversomers. *Tetrahedron Lett. 46*, 1611−1615.
(28) Mecca, T., Consoli, G. M. L., Geraci, C., and Cunsolo, F. (2004) Designed calix[8]arene-based ligands for selective tryptase surface recognition. *Bioorg. Med. Chem. 12*, 5057−5062.
(29) Chini, M. G., Terracciano, S., Riccio, R., Bifulco, G., Ciao, R., Gaeta, C., Troisi, F., and Neri, P. (2010) Conformationally locked calixarene-based histone deacetylase. *Org. Lett. 12*, 5382−5385.
(30) Consoli, G. M. L., Granata, G., Galante, E., Di Silvestro, I., Salafia, L., and Geraci, C. (2007) Synthesis of water-soluble nucleotide-calixarene conjugates and preliminary investigation of their in vitro DNA replication inhibitory activity. *Tetrahedron 63*, 10758−10763.
(31) Hwang, K. M., Qi, Y. M., Liu, S. Y., Lee, T. C., Choy, W., and Chen, J. (1996) U.S. Patent 5,409,959, 1995; *Chem. Abstr. 123*, 959c.
(32) Blaskovich, M. A., Lin, Q., Delarue, F. L., Sun, J., Park, H. S., Coppola, D., Hamilton, A. D., and Sebti, S. M. (2000) Design of GFB-111, a platelet-derived growth factor binding molecule with antiagiogenic and anticancer activity against human tumors in mice. *Nat. Biotechnol. 18*, 1065−1070.
(33) Dings, R. P., Miller, M. C., Nesmelova, I., Astorgues-Xerri, L., Kumar, N., Serova, M., Chen, X., Raymond, E., Hoye, T. R., and Mayo, K. H. (2012) Antitumor agent calixarene 0118 targets human galectin-1 as an allosteric inhibitor of carbohydrate binding. *J. Med. Chem. 55*, 5121−5129.
(34) Viola, S., Consoli, G. M. L., Merlo, S., Drago, F., Sortino, M. A., and Geraci, C. (2008) Inhibition of rat glioma cell migration and proliferation by a calix[8]arene scaffold exposing multiple GlcNAC and ureido functionalities. *J. Neurochem. 107*, 1047−1055.
(35) Mourer, M., Psychogios, N., Laumond, G., Aubertin, A.-M., and Regnouf-de-Vains, J.-B. (2010) Synthesis and anti-HIV evaluation of water-soluble calixarene-based bithiazolyl podands. *Bioorg. Med. Chem. 18*, 36−45.
(36) Tsou, L. K., Dutschman, G. E., Gullen, E. A., Telpoukhovskaia, M., Cheng, Y.-C., and Hamilton, A. D. (2010) Discovery of a synthetic dual inhibitor of HIV and HCV infection based on a tetrabutoxy-calix[4]arene scaffold. *Bioorg. Med. Chem. Lett. 20*, 2137−2139.
(37) Mourer, M., Fontanay, S., Duval, R. E., and Regnouf-de-Vains, J.-B. (2012) Synthesis, characterization, and biological evaluation as antibacterial agents of water-soluble calix[4]arenes and phenol derivatives incorporating carboxylate groups. *Helv. Chim. Acta 95*, 1373−1386.
(38) Consoli, G. M. L., Granata, G., Cafiso, V., Stefani, S., and Geraci, C. (2011) Multivalent calixarene-based fucosyl derivative: a new

Pseudomonas aeruginosa biofilm inhibitor. *Tetrahedron Lett. 52*, 5831−5834.
(39) Soomro, A. M., Oad, R. K., Memon, S., and Qureshi, I. (2012) Bioactivity assessment of water soluble calix[4]arene derivative. *Pak. J. Anal. Environ. Chem. 13*, 36−39.
(40) Consoli, G. M. L., Cunsolo, F., Geraci, C., Mecca, T., and Neri, P. (2003) Calix[8]arene-based glycoconjugates as multivalent carbohydrate-presenting systems. *Tetrahedron Lett. 44*, 7467−7470.
(41) Consoli, G. M. L., Cunsolo, F., Geraci, C., and Sgarlata, V. (2004) Synthesis and lectin binding ability of glycosamino acid-calixarenes exposing GlcNAc clusters. *Org. Lett. 6*, 4163−4166.
(42) Mecca, T., Consoli, G. M. L., Geraci, C. G., La Spina, R., and Cunsolo, F. (2006) Polycationic calix[8]arenes able to recognize and neutralize heparin. *Org. Biomol. Chem. 4*, 3763−3768.
(43) Testa, B., and Bojarski, A. J. (2000) Molecules as complex adaptative systems: constrained molecular properties and their biochemical significance. *Eur. J. Pharm. Sci. 11* (Suppl. 2), S3−S14.
(44) Ragupathi, G., Cappello, S., San, Yi, S., Canter, D., Spassova, M., Bornmann, W. G., Danishefsky, S. J., and Livingston, P. O. (2002) Comparison of antibody titers after immunization with monovalent or tetravalent KLH conjugate vaccines. *Vaccine 20*, 1030−1038.
(45) Woller, E. K., and Cloninger, M. J. (2002) The lectin-binding properties of six generations of mannose-functionalized dendrimers. *Org. Lett. 4*, 7−10.
(46) Kotera, Y., Fontenot, J. D., Pecher, G., Metzgar, R. S., and Finn, O. J. (1994) Humoral immunity against a tandem repeat epitope of human mucin MUC-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res. 54*, 2856−2860.
(47) Huang, Z.-H., Shi, L., Ma, J.-W., Sun, Z.-Y., Cai, H., Chen, Y.-X., Zhao, Y.-F., and Li, Y.-M. (2012) A totally synthetic, self-assembling, adjuvant-free MUC1 glycopeptide vaccine for cancer therapy. *J. Am. Chem. Soc. 134*, 8730−8733.