



Calix[n]arene-based immunogens: A new non-proteic strategy for anti-cocaine vaccine



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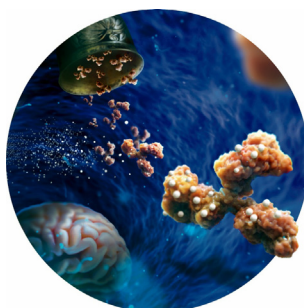
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HIGHLIGHTS

- The first total synthesis of the novel calix[n]arene-based immunogens **V4N2** and **V8N2** is reported.
- **V4N2** and **V8N2** promoted the production of cocaine antibodies and also modulated the biodistribution of [^{99m}Tc]TRODAT-1, a radiolabeled analogue of cocaine.
- **V4N2** and/or **V8N2** are potential candidates for the development of an immunogenic agent for the treatment of cocaine use disorder.

GRAPHICAL ABSTRACT

Two novel calix[n]arene-based immunogens were able to yield anti-cocaine antibodies and decrease the levels of [^{99m}Tc]TRODAT-1, a cocaine-based radionuclide, in the brain of the tested mice.



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ABSTRACT

Introduction: Cocaine use disorder is a significant public health issue without a current specific approved treatment. Among different approaches to this disorder, it is possible to highlight a promising immunologic strategy in which an immunogenic agent may reduce the reinforcing effects of the drug if they are able to yield sufficient specific antibodies capable to bind cocaine and/or its psychoactive metabolites before entering into the brain. Several carriers have been investigated in the anti-cocaine vaccine development; however, they generally present a very complex chemical structure, which potentially hampers the proper assessment of the coupling efficiency between the hapten units and the protein structure.

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Crack
Chemical addiction
Calixarenes
Immunotherapy
TRODAT-1

Objectives: The present study reports the design, synthesis and preclinical evaluation of two novel calix[n]arene-based anti-cocaine immunogens (herein named as **V4N2** and **V8N2**) by the tethering of the hydrolysis-tolerant hapten **GNE (15)** on calix[4]arene and calix[8]arene moieties.

Methods: The preclinical assessment corresponded to the immunogenicity and dose–response evaluation of **V4N2** and **V8N2**. The potential of the produced antibodies to reduce the passage of cocaine analogue through the blood–brain-barrier (BBB), modifying its biodistribution was also investigated.

Results: Both calix[n]arene-based immunogens elicited high titers of cocaine antibodies that modified the biodistribution of a cocaine radiolabeled analogue (^{99m}Tc -TRODAT-1) and decreased cocaine-induced behavior, according to an animal model.

Conclusion: The present results demonstrate the potential of **V4N2** and **V8N2** as immunogens for the treatment of cocaine use disorder.

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Introduction

Cocaine use disorder (CUD) is a persistent public health problem with no single “best” pharmacological agent for its treatment [1–4]. Despite of the efforts to educate the population about the dangers of the use of cocaine, CUD remains a major social and health challenge [5–10]. Increment in the number of cocaine users from 14 million in 1998 to 18 million in 2017, was noted by the United Nations Office on Drugs and Crime, which corresponds to nearly 0.4% of the world population aged 15–64 [11]. Brazil occupies the first and second places in the rank of crack consumption (smoked form of cocaine) and cocaine traffic, respectively [12–15].

The international scientific community has been struggling to find an effective treatment for CUD. Currently, the available pharmacological treatments are only used to manage withdrawal syndrome and relapse prevention [16,17]. Given the range of therapies for CUD, the development of an immunologic strategy is a promising approach [18]. In fact, immunogenic agents may reduce the reinforcing effects of the drug if they are able to yield high titers of highly specific antibodies capable to bind cocaine and/or its psychoactive metabolites before entering into the brain. As this immune mechanism is similar to the one observed in antiviral or antibacterial vaccines, this strategy was popularly named “anti-drug vaccine”. This concept has been previously demonstrated to be successful in blunting the physiological effects of cocaine [18–25], methamphetamine [26–30], heroin [31–34], opioids [35–37], nicotine [38–41], etc.

Cocaine is barely not immunogenic, so, to become immunogenic, cocaine should be chemically modified into a derivative called hapten, which can subsequently be conjugated to a macromolecular carrier prior to the administration [18–25]. Both *N*-methyl and 2 β methyl ester groups of cocaine have been extensively targeted to synthesize haptens able to link to a carrier [9,42–48]. For instance, GNE (6-(1*R*,2*R*,3*S*,5*S*)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2 carboxamide-hexanoic acid), a hydrolysis-tolerant hapten, stimulates the production of cocaine-specific antibodies when carried by high molecular weight proteins [25,46,49–51].

Several carriers have been investigated in the anti-cocaine vaccine development [48] such as viral-DNA fragments/proteins [25,49,52], peptide nanofiber [53] and proteins, like the subunit of cholera toxin [21], flagellin [54] and keyhole limpet hemocyanin (KLH) [43]. Peptide-based carriers have present a very complex chemical structure, which potentially hampers the assessment of the coupling efficiency between the hapten units and the protein structure [55]. Moreover, most synthetic methodologies to couple the hapten to the protein employ the use of a carrier in excess. Once in excess, the peptide-based carrier could increase the production of antibodies against the carrier itself and, consequently, decrease the desired immunogenicity of the exposed hapten [55–57]. In addition, protein-based vaccines require a complex system

for sterilizations and a cold supply chain, considering the possible risk of denaturalization of proteins when exposed to heat.

Viruses and bacteria display a well-organized, repetitive array of antigens on surface, which is believed to successfully allow the clustering of antigen receptors on B cells. This phenomenon boosts the production of antibodies in comparison with the interaction between these antigens with soluble proteins [58–60]. These findings suggested that antigens’ subunit immunogenicity is improved when the antigens are rigidly ordered on surfaces, similarly to the observed for viral particles [58,60]. Therefore, this multivalency of antigen arrangement, together with the easing of immune cell recognition and antigen incorporation, has been explored as an approach to improve both humoral and cellular immunity [60].

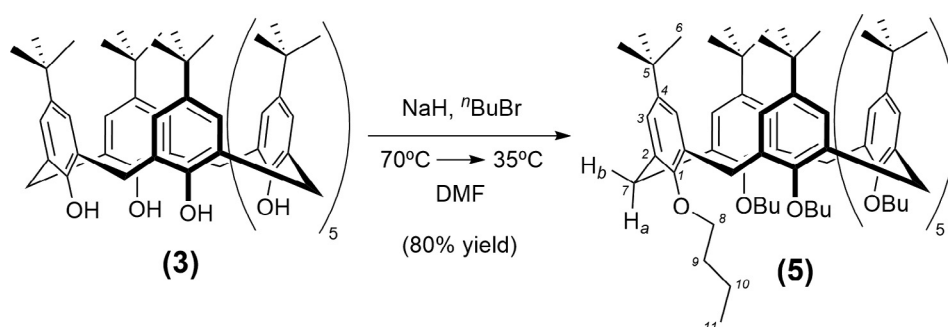
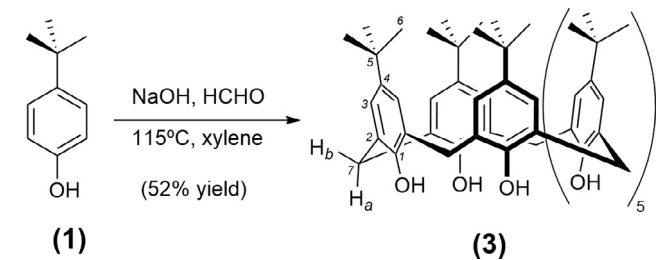
Considering the difficulty in coupling efficiency verification between haptens and biomacromolecules, the antibody production against the carriers due to excess in synthesis, and the importance of spatially organized antigens, we envisioned the use of calix[n]arene as a potential carrier for the development of cocaine immunogens. To the best of our knowledge, cocaine calix[n]arene-based immunogens are unprecedented, and such macrocycles were used as carriers only for the development of an anti-cancer vaccine, firstly reported by Geraci and collaborators in 2008, thus far [61]. According to these authors, the high antibody titer yield in their study, is a consequence of the three-dimensional arrangement adopted by the calix[4]arene platform. Subsequently, in another study, this same research group observed that a calix[8]arene-based anticancer vaccine candidate also induced a specific immune response, ratifying the promising applicability of calix[n]arenes in building vaccine constructs [62]. Thus, the present study reports the design, synthesis and preclinical evaluation of two novel calix[n]arene-based anti-cocaine immunogens (herein named as **V4N2** and **V8N2**; Scheme 3) by the tethering of the hydrolysis-tolerant hapten GNE on calix[4]arene and calix[8]arene moieties. The preclinical assessment corresponded to the immunogenicity and dose–response evaluation of **V4N2** and **V8N2**. The potential of the produced antibodies to reduce the transport of the cocaine analogue ^{99m}Tc -TRODAT-1 through the blood–brain-barrier (BBB), modifying its biodistribution was also investigated.

Materials and Methods

Synthesis procedures

5,11,17,23-tetra-tert-Butyl-25,26,27,28-tetrahydroxycalix[4]arene (2), **5,11,17,23-Tetra-tert-butyl-25,26,27,28-tetrabutoxyca**
lix[4]arene (4), **5,11,17,23-Tetranitro-25,26,27,28-tetrabutoxyca**
lix[4]arene (6) and **5,11,17,23-Tetraamine-25,26,27,28-tetrabu**
toxyca**lix[4]arene (8)** were previously synthesized by our research

group and their spectroscopic data are in accordance with those published elsewhere [63]. IR, ^1H - and ^{13}C and HRMS for compounds **2**, **4**, **6** and **8** are available as a Supplementary Material.

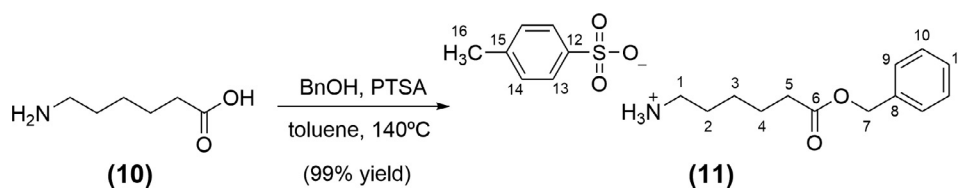
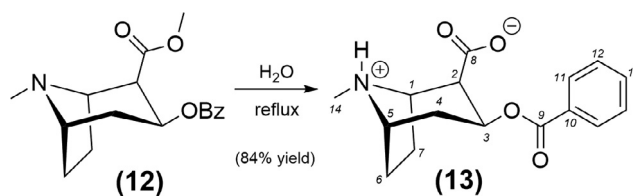


5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55,56-octahydroxycalix[8]arene (3). A mixture containing *p*-tert-butylphenol (10.00 g; 66.5 mmol), paraformaldehyde (3.60 g; 120 mmol), 1.5 mL of a 10 mol L⁻¹ sodium hydroxide solution and 300 mL of xylene was additionally in a round-bottomed three-necked flask and, after, it was mechanically stirred for approximately 15 min. Subsequently was coupled to a Dean-Stark apparatus containing 100 mL of xylene. The mixture was refluxed at 115 °C by using a heating mantle, with continuous flow of nitrogen, for approximately 4 h. After 30 min of heating, the reaction became a homogeneous mixture, and after 1 h, a white precipitate begins to form. The mixture was cooled to room temperature and the precipitate formed was collected by filtration. The precipitate was washed successively with 200 mL of toluene, 200 mL of ethyl ether, 200 mL of acetone and finally with

200 mL of distilled water. After that, then dried under reduced pressure. The solid obtained was dissolved in 800 mL of chloroform and heated until its volume was reduced to 600 mL. The solution was then cooled to room temperature and the precipitate formed was then collected and dried. This methodology provides the desired product **3** as a white solid (10.50 g) in 52% yield. **IR (ATR):** 3225, 3052, 2955, 2902, 2868, 1602, 1486, 1452, 1427, 1391, 1361, 1290, 1247, 1203, 1149, 1117 and 783 cm⁻¹. **$^1\text{H NMR}$** (300 MHz, CDCl₃): δ 9.62 (s, 8H, OH), 7.18 (s, 16H, H₃), 4.37 (d, 8H, $J_{7a,7b}$ = 12.0 Hz, H_{7a}), 3.50 (d, 8H, $J_{7b,7a}$ = 12.0 Hz, H_{7b}), 1.26 (s, 72H, H₆). **$^{13}\text{C NMR}$** (75 MHz, CDCl₃): δ 146.6 (C₁), 144.7 (C₂), 128.7 (C₄), 125.5 (C₃), 34.0 (C₇), 32.3 (C₅), 31.4 (C₆). **HRMS (MALDI-TOF)** calcd. for C₈₈H₁₁₃O₈ [M+H]⁺ 1,298.8340; found 1,298.8239.

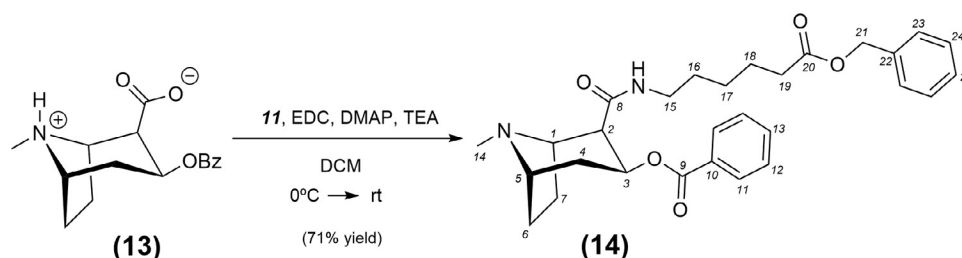
5,11,17,23,29,35,41,47-Octa-tert-butyl-49,50,51,52,53,54,55,56-octabutoxycalix[8]arene (5). In a round-bottomed flask (500 mL) with a magnetic bar, a mixture containing compound **3** (1.00 g, 0.77 mmol) and dry dimethylformamide (150 mL), under stirring, was heated to 70 °C until a translucent solution was obtained. After that, were added 6 g of dispersed NaH in 60% of oil solution. The mixture remained under stirring at 60 °C for 1 h. Subsequently, was added 50 mL of 99% 1-bromobutane (77 mmol) and potassium iodide (5.00 g; 30 mmol). The temperature of the mixture was lowered to 35 °C, and the reaction remained under stirring for 72 h. After this time, 100 mL of dichloromethane were added and the mixture was washed with 200 mL of 0.1 mol L⁻¹ HCl solution. The organic phase was concentrated *in vacuo* until half of the amount of solvent added was evaporated. After that, 200 mL of acetone were added, and the mixture was again concentrated *in vacuo* until a white precipitate was formed. The precipitate was recovered by filtration with a Büchner funnel and washed with

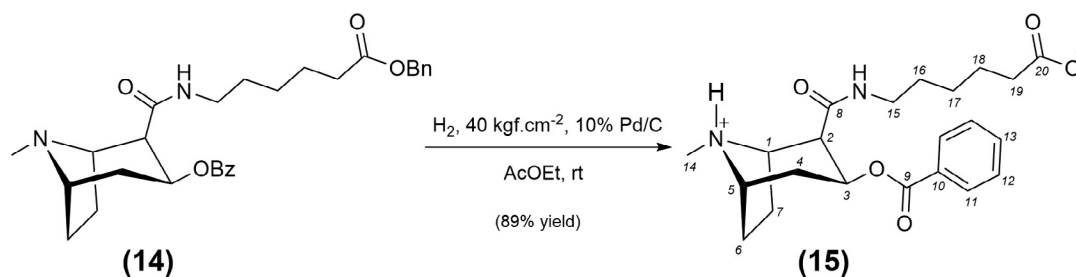
2930, 2868, 1606, 1458, 1379, 1210, 1124, 1082, 1064, 1019, 955 and 853 cm^{-1} . $^1\text{H NMR}$ (200 MHz, CDCl_3 and $\text{DMSO } d_6$): δ 6.17 (s, 16H, H_3), 3.83 (s, 16H, H_7), 3.77 (t, 16H, $J_{8,9} = 6.0$ Hz, H_8), 3.51 (s, 24H, NH_2 e H_2O), 1.62–1.90 (m, 16H, H_9), 1.34–1.62 (m, 16H, H_{10}), 0.95 (t, 24H, $J_{11,10} = 6.0$ Hz, H_{11}). $^{13}\text{C NMR}$ (50 MHz, CDCl_3 and $\text{DMSO } d_6$): δ 147.5 (C_1), 142.0 (C_4), 134.8 (C_2), 115.2 (C_3), 73.5 (C_8), 32.2 (CH_2), 29.2 (CH_2), 19.1 (C_{10}), 13.8 (C_{11}). **HRMS** (MALDI-TOF) calcd. for $\text{C}_{88}\text{H}_{121}\text{N}_8\text{O}_8$ $[\text{M}+\text{H}]^+$ 1,418.9511; found 1,418.9326.



6-Amino-hexanoic acidbenzylester toluene-4-sulfonic acid (11). In a flask with 6-amino-hexanoic acid (1.32 g, 10.1 mmol) were added benzyl alcohol (3.82 g, 35.3 mmol), *p*-toluenesulfonic acid monohydrate (2.01 g, 10.6 mmol) and 25 mL of toluene. The reaction mixture was warmed and refluxed for 24 h with a *Dean-Stark* apparatus. After cooling to room temperature, the precipitate was filtered, washed with diethyl ether (2×25 mL) and dried to furnish 3.93 g of the desired product **11** (99% yield). **IR (ATR):** 3262, 3186, 3043, 2949, 2869, 1725, 1479, 1450, 1304, 1262, 1249, 1194, 1161, 1142, 1127, 1068, 1037, 1013, 960, 919, 824, 751 and 683 cm^{-1} . $^1\text{H NMR}$ (200 MHz, CDCl_3): δ 7.75–7.66 (m, 5H, H_{13} and NH_3), 7.32 (brs, 5H, H_9 , H_{10} and H_{11}), 7.15 (d, 2H, $J_{14,13} = 8.0$ Hz, H_{14}), 5.07 (s, 2H, H_7), 2.75–2.73 (m, 2H, H_1), 2.31 (s, 3H, H_{16}), 2.20 (t, 2H, $J_{5,4} = 8.0$ Hz, H_5), 1.49–1.43 (m, 4H, H_2 and H_3), 1.20–1.16 (m, 2H, H_4). $^{13}\text{C NMR}$ (50 MHz, CDCl_3): δ 173.3 (C_6), 141.4 (C_{Ar}), 141.0 (C_{Ar}), 136.2 (C_8), 129.2 (CH_{Ar}), 128.7 (CH_{Ar}), 128.3 (CH_{Ar}), 126.0 (CH_{Ar}), 66.3 (C_7), 39.9 (CH_2), 34.0 (CH_2), 27.2 (CH_2), 25.9 (CH_2), 24.3 (CH_2), 21.4 (C_{16}).

Benzoylcocaine (13). 1.17 g (3.87 mmol) of cocaine (**12**) was refluxed and stirred in water distilled (20 mL) for 24 h. The solvent was removed under reduced pressure and acetone (80 mL) was added into crude product. After stirred in an ice bath, the desired product was obtained as a white powder in 84% yield (0.94 g). **IR (ATR):** 3224, 1718, 1590, 1476, 1449, 1401, 1351, 1270, 1196, 1115, 1076, 1025, 996, 983, 810, 789 and 708 cm^{-1} . $^1\text{H NMR}$ (200 MHz, CD_3OD): δ 8.05–8.01 (m, 2H, H_{11}), 7.64–7.57 (m, 1H, H_{13}), 7.46 (t, 2H, $J_{1,3} = 8.0$ Hz, H_{12}), 5.48 (dt, 1H, $J_{3,4a} = 12.0$ Hz, $J_{3,4b} = J_{3,2} = 6.0$ Hz, H_3), 4.00–3.95 (m, 2H, H_1 and H_5), 3.10 (dd, 1H, $J_{2,3} = 6.0$ Hz, $J_{2,1} = 2.0$ Hz, H_2), 2.81 (s, 3H, H_{14}), 2.65–2.10 (m, 6H, H_4 , H_6 and H_7). $^{13}\text{C NMR}$ (50 MHz, CD_3OD): δ 177.0 (C_8), 167.2 (C_9), 134.6 (C_{13}), 131.3 (C_{10}), 131.0 (C_{11}), 129.6 (C_{12}), 66.7 (CH), 66.0 (CH), 63.7 (CH), 50.3 (CH), 38.3 (C_{14}), 34.4 (CH_2), 25.1 (CH_2), 24.7 (CH_2). **HRMS** (ESI) calcd. for $\text{C}_{16}\text{H}_{19}\text{NO}_4\text{Na}$ $[\text{M}+\text{Na}]^+$ 312.1212; found 312.1104.





(1R,2R,3S,5S)-2-((6-(Benzyloxy)-6-oxohexyl)carbamoyl)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl Benzoate (14). To a solution of benzoyllecgonine **13** (1.16 g, 4.0 mmol), 6-amino-hexanoic acid benzyl ester toluene-4-sulfonic acid (1.57 g, 4.0 mmol) and 4-dimethylaminopyridine (0.50 g, 4.0 mmol) in dry dichloromethane (20 mL) were added EDC (0.86 g, 4.4 mmol) and triethylamine (0.56 mL, 4.0 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 96 h in a inert atmosphere (Ar). The organic layer was washed with 10% citric acid solution (3 × 15 mL), water (1 × 20 mL) and brine (1 × 20 mL), dried with anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude product was purified in a chromatography column using ethyl acetate/hexan/Et₃N (30:10:4, v/v) as mobile phase and the desired product was obtained in 71% yield (1.39 g). **IR (ATR):** 2932, 2857, 1718, 1663, 1544, 1451, 1267, 1228, 1155, 1112, 1069, 1045, 1027, 988, 735, 712, 698 and 678 cm⁻¹. **¹H NMR** (200 MHz, CDCl₃): δ9.54 (brs, 1H, NH), 7.98 (d, 2H, *J* = 6.0 Hz, *H*₁₁), 7.52–7.28 (m, 8H, *H*₁₂, *H*₁₃, *H*₂₃, *H*₂₄ and *H*₂₅), 5.31 (dt, 1H, *J*_{3,4a} = 12.0 Hz, *J*_{3,4b} = *J*_{3,2} = 5.0 Hz, *H*₃), 5.10 (s, 2H, *H*₂₁), 3.42–3.19 (m, 4H), 2.93–2.87 (m, 1H), 2.37–1.88 (m, 9H), 1.72–1.36 (m, 8H). **¹³C NMR** (50 MHz, CDCl₃): δ173.1 (C=O), 170.8 (C=O), 165.7 (C=O), 135.9 (C₂₂), 132.7 (CH_{Ar}), 130.1 (C₁₀), 129.5 (CH_{Ar}), 128.4 (CH_{Ar}), 128.1 (CH_{Ar}), 128.0 (CH_{Ar}), 65.9 (C₂₁), 65.8 (CH), 63.1 (CH), 60.2 (CH), 51.0 (CH), 40.3 (C₁₄), 38.6 (CH₂), 35.7 (CH₂), 33.9, (CH₂), 29.4 (CH₂), 26.4 (CH₂), 25.7 (CH₂), 24.7 (CH₂), 24.5 (CH₂). **HRMS** (ESI) calcd. for C₂₉H₃₇N₂O₅ [M+H]⁺ 493.2702; found 493.2688.

6-((1R,2R,3S,5S)-3-(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxamido)hexanoic Acid (GNE). A mixture of compound **14** (1.35 g, 2.7 mmol) and 10% Pd/C (0.22 g) in 40 mL of ethyl acetate was stirred for 96 h at room temperature under H₂ pressure. The catalyst was removed by filtration using Celite® and concentrated under diminished pressure to give the product **GNE** in 89% yield (0.98 g). **IR (ATR):** 3255, 3062, 2938, 2861, 1716, 1638, 1553, 1491, 1451, 1395, 1347, 1316, 1269, 1197, 1112, 1070, 1046, 1026, 998, 804 and 711 cm⁻¹. **¹H NMR** (200 MHz, CD₃OD): δ7.97 (d, 2H, *J* = 8.0 Hz, *H*₁₁), 7.63 (t, 1H, *J* = 8.0 Hz, *H*₁₃), 7.48 (t, 2H, *J* = 7.0 Hz, *H*₁₂), 5.57–5.44 (m, 1H, *H*₃), 4.00–3.99 (m, 1H), 3.88–3.87 (m, 1H), 3.23–3.14 (m, 3H), 2.73 (s, 3H, *H*₁₄), 2.52–2.01 (m, 8H), 1.58–1.20 (m, 7H). **¹³C NMR** (50 MHz, CD₃OD): δ181.4 (C₂₀), 173.1 (C₈), 166.8 (C₉) 134.9 (C₁₃), 130.9 (C₁₀), 130.8 (C₁₁ and C₁₂), 129.8 (C₁₁ and C₁₂), 66.2 (CH), 66.1 (CH), 63.8 (CH), 48.3 (CH), 40.5 (CH₂), 39.3 (C₁₄), 38.1 (CH₂), 35.0 (CH₂), 30.2, (CH₂), 28.0 (CH₂), 27.0 (CH₂), 25.4 (CH₂), 24.8 (CH₂). **HRMS** (ESI) calcd. for C₂₂H₃₁N₂O₅ [M+H]⁺ 403.2233; found 403.2212.

Immunogenic compound V4N2. The hapten **GNE (15)** (0.98 g; 2.43 mmol) was dissolved in dry dichloromethane (8 mL) and reacted with PyBOP (1.58 g; 3.04 mmol) and DIPEA (0.31 g; 2.43 mmol). After stirring for 40 min at room temperature, a solution of calixarene **8** in dry dichloromethane (10 mL) was added dropwise in inert atmosphere (Ar). The mixture was kept under stirring for 26 h and then was added dichloromethane (80 mL). The organic layer was washed with sat. aq. NaHCO₃ (2 × 25 mL) and water (1 × 25 mL), dried over anhydrous sodium sulfate, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography (CHCl₃/MeOH/TEA, 93:6:1, v/v) to provide the desired product **V4N2** (0.60 g; 59% yield) as a yellow solid. **IR (ATR):** 3260, 2934, 2864, 1716, 1651, 1600, 1539, 1467, 1451, 1415, 1352, 1267, 1212, 1176, 1113, 1069, 1027, 984, 867, 803, 748, 710 and 687 cm⁻¹. **¹H NMR** (600 MHz, CDCl₃): δ9.60 (brs, 4H, NH), 7.97 (d, *J* = 6.0 Hz, 8H, *H*₂₉), 7.44–7.35 (m, 12H, *H*₃₀, *H*₃₁), 6.76 (brs, 4H, *H*₃), 6.75 (brs, 4H, *H*₃), 5.31 (dt, 4H, *J*_{21,22a} = 10.2 Hz, *J*_{21,22b} = *J*_{21,20} = 6.6 Hz, *H*₂₁), 4.38 (d, 4H, *J* = 13.2 Hz, *H*_{7a}), 3.84 (brs, 8H, *H*₈), 3.35–3.27 (m, 16H, *H*₁₉, *H*₁₇ and *H*₂₃), 3.06 (d, 4H, *J* = 13.2 Hz, *H*_{7b}), 2.90 (d, 4H, *J* = 6.6 Hz), 2.25 (s, 12H, *H*₂₆), 2.21–2.06 (m, 20H, *H*_{25a}, *H*_{24a}, *H*_{22a} and *H*₁₃), 1.99–1.37 (m, 52H, *H*_{22b}, *H*₉, *H*_{24b}, *H*_{25b}, *H*₁₄, *H*₁₆, *H*₁₀ and *H*₁₅), 1.00 (t, 12H, *J* = 7.2 Hz, *H*₁₁). **¹³C NMR** (100 MHz, CDCl₃): δ171.5 (C₁₂), 171.2 (C₁₈), 166.1 (C₂₇), 153.5 (C₁), 135.2 (C₂), 133.1 (C₃₁), 132.0 (C₄), 130.4 (C₂₈), 129.8 (C₂₉), 128.5 (C₃₀), 121.4 (C₃), 75.1 (C₈), 66.2 (C₂₁), 63.4 (C₁₉), 60.6 (C₂₃), 51.3 (C₂₀), 40.6 (C₂₆), 38.9 (C₁₇), 37.0 (C₁₃), 36.0 (C₂₂), 32.3 (C₉), 31.2 (C₇), 29.6 (C₁₆), 26.6 (C₁₅), 26.0 (C₂₅), 25.3 (C₁₄), 25.0 (C₂₄), 19.5 (C₁₀), 14.2 (C₁₁). **HRMS** (MALDI-TOF) calcd. for C₁₃₂H₁₇₃N₁₂O₂₀ [M+H]⁺ 2,247.2917; found 2,247.2908.

Immunogenic compound V8N2. In a round-bottomed flask (100 mL), with a magnetic bar, was added the hapten **GNE** (0.710 g, 1.76 mmol), dry dichloromethane (10 mL), PyBOP (1.04 g, 2.0 mmol) and DIPEA (0.31 g, 2.43 mmol). The mixture was stirring for 40 min, at room temperature. After that, a solution of calixarene **9** (0.80 g, 0.056 mmol), in dry dichloromethane (10 mL), was added dropwise. The mixture was kept under hard stirring for 48 h in inert atmosphere. The mixture was subjected to two purifications: one with Sephadex LH-20 (in CH₂Cl₂) and another with silica-gel chromatography (CHCl₃/MeOH/TEA, 94:5:1 v/v), to provide the desired product **V8N2**, as a yellow pale solid, in 60% yield (200 mg). **IR (ATR):** 3261 and 3193 cm⁻¹ (CH of aromatic ring stretching), 2932 and 2863 cm⁻¹ (CH of methyl and methylene groups stretching), 1716 cm⁻¹ (carbonyl ester stretching), 1649 cm⁻¹ (carbonyl amide stretching), 1542 cm⁻¹ (N-H amide angular deformation), 1267 cm⁻¹ (C–O stretching),

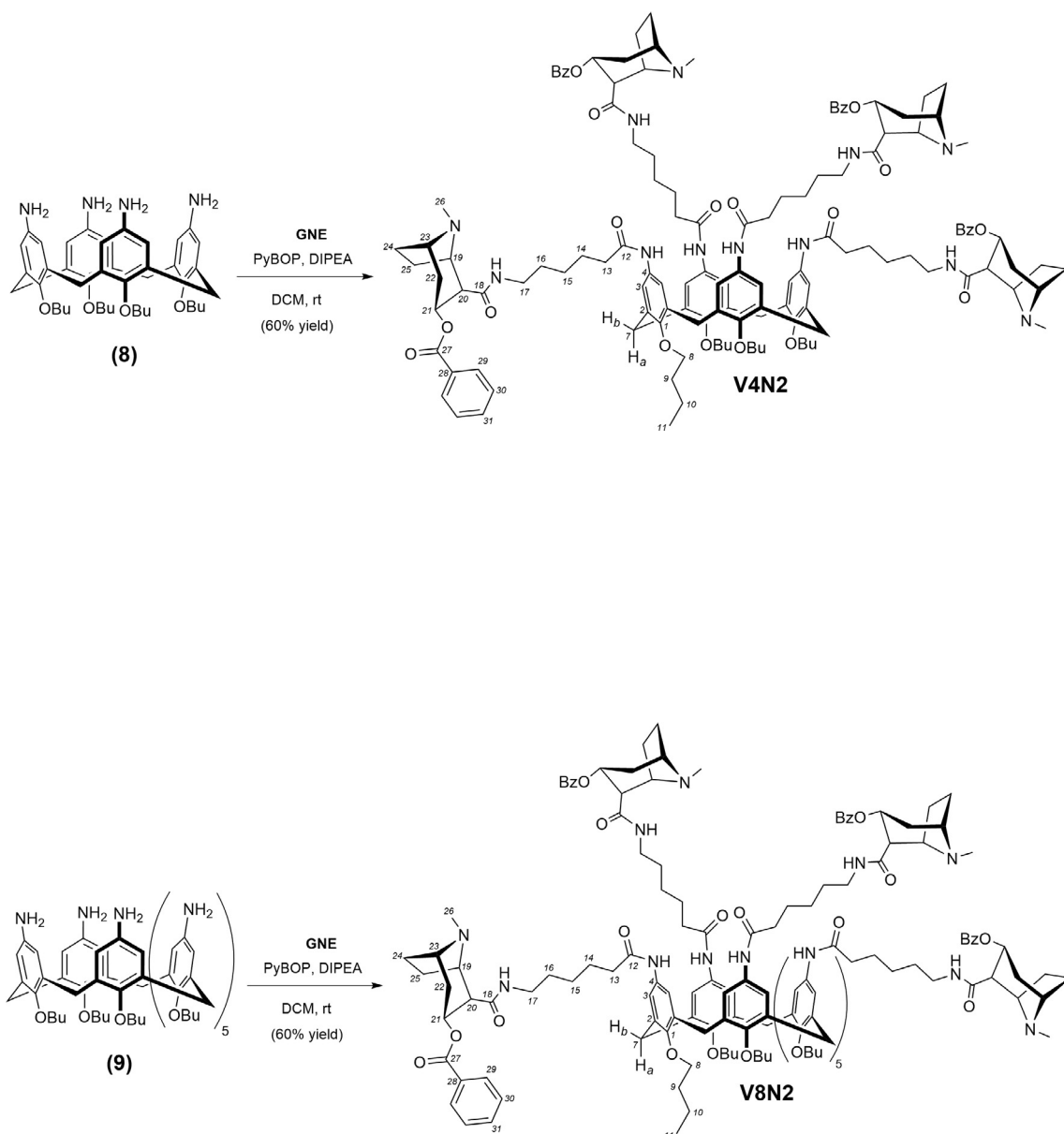
710 cm^{-1} (C=C angular deformation). $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 9.57 (brs, 8H, NH), 7.95 (d, $J_{29,30/31} = 8.0$ Hz, 16H, H_{29}), 7.47 (t, $J_{30,31} = 8.0$ Hz, 8H, H_{31}), 7.30–7.40 (m, 16H, H_{30}), 7.01 (brs, 16H, H_3), 5.18–5.36 (m, 8H, H_{21}), 3.94 (brs, 16H, H_7), 3.76–4.16 (m, 8H, H_{8b}), 3.40–3.76 (m, 8H, H_{8a}), 3.01–3.49 (m, 32H, H_{19} , H_{17a} , H_{17b} , H_{23}), 2.61–2.79 (m, 8H, H_{20}), 2.17 (s, 24H, H_{26}), 2.10–2.29 (m, 40H, H_{22a} , H_{24b} , H_{25b} e H_{16}), 1.84–2.10 (m, 8H, H_{22b}), 1.46–1.84 (m, 80H, H_{25a} , H_{24a} , H_{14} , H_{13} , H_{10} e H_9), 1.25 (brs, 16H, H_{15}), 0.83 (brs, 24H, H_{11}). $^{13}\text{C NMR}$ (100 MHz, CDCl_3): δ 171.7 (C_{18}), 171.1 (C_{12}), 165.9 (C_{27}), 151.9 (C_1), 134.0 (C_4), 133.9 (C_2), 132.9 (C_{31}), 130.3 (C_{28}), 129.7 (C_{29}), 128.3 (C_{30}), 121.6 (C_3), 73.0 (C_8), 66.0 (C_{21}), 63.2 (C_{19}), 60.4 (C_{23}), 51.1 (C_{20}), 46.1 (CH_2), 40.3 (C_{26}), 38.8 (C_{17}), 35.8 (C_{22}), 32.3 (C_9 and C_7), 29.4 (C_{13}), 25.8 (CH_2), 25.3 (CH_2), 24.3 (CH_2), 19.3 (CH_2), 14.0 (C_{11}). **HRMS** (MALDI-TOF) calcd. for $\text{C}_{264}\text{H}_{345}\text{N}_{24}\text{O}_{40}$ $[\text{M}+\text{H}]^+$ 4,494.7009; found 4,494.6128.

Animal studies

The Local Ethics Committee in Animal Experimentation approved the study protocol (CEUA-UFMG Protocol N $^\circ$. 122/2016) and at all stages of the work, the recommendations of this commission were adopted.

Preparation of immunoconjugate hapten 15-KLH

2.0 mg of KLH protein were reconstituted with 200 μL of water ultra-purified and it was stored in an ice bath. Then, 2.0 mg of the GNE hapten (**15**) were diluted in 450 μL of the conjugation buffer (MES 0.1 M, 0.9 M NaCl, 0.02% sodium azide, pH 4.7), stirred until complete solubilization of the hapten and added to the aqueous solution containing the protein KLH. EDC coupling agent (10.0 mg) was reconstituted with 1000 μL of ultra-purified water



and quickly stirred. Immediately, 50 μL of this solution were transferred to the solution containing the protein and the hapten **15** and kept at room temperature for two hours. Then the brute of the reaction was purified on a desalination column (polypropylene resin) by centrifugation (1000g for 2 min), providing 700 μL of the immunoconjugate hapten **15**-KLH.

Preparation of immunogen's formulation

Calix[n]arene-based immunogen **V4N2** or **V8N2**: In a 15 mL Falcon[®] tube containing an appropriate amount of **V4N2** or **V8N2** were added 125 μL of the dimethylsulfoxide (DMSO). Then 2,435 μL of Freund's Adjuvant was added (Complete or Incomplete) and 2,435 μL of saline solution. The two-phase system was then subjected to a 3000 rpm agitation in Vortex[®] for 20 min. The emulsion at final concentration of 0.03, 3.0 or 300 μM of **V4N2** or **V8N2** was immediately stored on ice until time of administration.

Immunoconjugate hapten **15**-KLH: In a 15 mL Falcon[®] tube containing 175 μL of the immunoconjugate hapten **15**-KLH added 2500 μL of Freund's Adjuvant (Complete or Incomplete) and 2500 μL of sterile saline solution. The system biphasic was then subjected to an immunoconjugate hapten **15**-KLH stirring of 3000 rpm in Vortex[®] for 20 min. The emulsion was immediately stored on ice until the time of administration.

The vehicle for the immunogen's formulation was prepared as following: In a 15 mL Falcon[®] tube containing 125 μL of DMSO were added 2435 μL of Freund's Adjuvant (Complete or Incomplete) and 2435 μL of sterile saline solution. The two-phase system was then submitted at 3000 rpm agitation in Vortex[®] for 20 min. The emulsion was immediately stored on ice until the time of management.

Animals and blood sample collections

Male BALB/c mice aged 4 to 5 weeks were used. The animals were kept on a 12-h light–dark cycle with chow and water provided *ad libitum*. All experiments were carried out in a room with an ambient temperature of 23 ± 1 °C. Blood samples were collected by the puncture method of the submandibular vein into 1.5 mL Eppendorf[®] tubes containing the anticoagulant ethylenediamine tetraacetic acid (EDTA; 0.5 M; 6 μL /tube) and immediately centrifuged at 3000 rpm, at temperature of 4 °C for 25 min. Then, the plasma samples were separated and transferred to new Eppendorf[®] tubes (0.5 mL) and stored in a freezer at -30 °C until performing the immunoenzymatic assay.

Animal immunization scheme

Sixty male BALB/c mice (divided into five groups with twelve animals each) were immunized on days 0, 7 and 21. On each endpoint, the animals received *i.p.* 300 μL of the emulsion containing **V4N2** or **V8N2** (30 nM; 3 μM or 300 μM) or 300 μL of the emulsion containing vehicle alone or 300 μL of the emulsion containing the KLH-GNE immunoconjugate.

Enzyme-linked immunosorbent assay (ELISA)

Anti-cocaine IgG antibodies was assessed as described by Fetisov (2011) [64]. The method used Maxisorp plates (Nunc, Rochester, NY) coated with 100 μL in each well of a solution 2 mg/mL of cocaine hydrochloride diluted in a buffer constituted of 0.05 M Na_2CO_3 , 0.05 M NaHCO_3 , 0.02% sodium azide (pH 9.6) for 24 h at 4 °C. Plates were washed three times in phosphate-buffered saline (PBS) with Tween-20 0.05 % (Sigma-Aldrich, Saint Louis, MO, USA), pH 7.4. Plasma was diluted 1:200 in sample buffer (PBS, 0.02 % sodium azide) and incubated overnight at 4 °C in the plate, with 100 μL in each well. The plates were washed three times and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:2000; 100 μL) (Sigma, St. Louis, MO, USA) in each well for 3 h

at 37 °C. After washing, 100 μL of 4-nitrophenyl phosphate disodium salt hexahydrate solution, diluted as recommended by the manufacturer (Sigma, St. Louis, MO, USA), was added to each well. After 40 min of incubation at room temperature (RT) in the dark, the reaction was stopped with 50 μL of 3 N NaOH (STOP solution) in each well. The optical density (OD) was determined at 405 nm using a VICTOR X4 multilabel plate reader (PerkinElmer[®]) (Fig. 1. A-B, Fig. 2. A-B).

Radiochemical assay

^{99m}Tc-TRODAT-1 was both prepared and the radiochemical purification was evaluated as recommended by the manufacturer (RPH Pharma, Porto Alegre, Brasil). Male Balb/c mice immunized with calixarene-based immunoconjugates **V4N2** or **V8N2** or placebo on days 0, 7 and 21 at the dose of 30 nM. Antibodies IgG anti-cocaine levels was assessed by ELISA method as described before. At 40th day, animals were submitted at radiochemical assay. 0.1 mL of the ^{99m}Tc-TRODAT-1 was administered in the tail vein of each animal. After 90 min, we acquired scintigraphic images of the brain region of each animal in a gamma camera scan (Nuclide TM H 22, Mediso, Hungria) (Fig. 4A). Animals were euthanized immediately after image acquisition and the radioactivity levels were determined in blood, brain (Fig. 4B), spleen, liver, lung, kidney, heart and stomach (Fig. 4C).

Statistical analysis

Statistical analyzes were performed using the GraphPad program Prism, version 5.01 (San Diego, CA, USA). The results were evaluated by the Kolmogorov–Smirnov normality test and the presence of outliers was detected by the Grubbs test. The results regarding the biodistribution of the [^{99m}Tc]TRODAT-1 were analyzed by Student's *t* test and presented by mean and standard error. In evaluating the production of antibodies between the groups, data were analyzed using simple analysis of variance, followed by the Bonferroni test. Values of $p \leq 0.05$ were considered as a difference statistically significant.

Results and discussion

Our initial efforts to obtain cocaine calix[n]arene-based immunogens focused on the preparation of calix[n]arenes **8** and **9** (Scheme 1). Firstly, the treatment of *p*-*tert*-butylphenol (**1**) under basic conditions at appropriate temperatures and solvents [65–67] provided the corresponding *p*-*tert*-butyl-calix[4]arene (**2**) and *p*-*tert*-butyl-calix[8]arene (**3**) in 51% and 52% yield, respectively. Then, alkylation reactions of the phenolic hydroxyl groups of **2** [63,68] and **3** [69] were performed using butyl bromide in the presence of sodium. However, in the case of calix[8]arene **3**, the *per-O*-alkylation was only observed when potassium iodide (KI) was used as an additive. The addition of KI to promote a *per-O*-alkylation of **3** with propyl bromide was also reported by Yi et al (2008) [69]. Thus, compounds **4** and **5** were synthesized in 77% and 80% yields, respectively. [69]. The *ipso*-nitration of **4** to furnish **6** in 91% yield was performed using a mixture of fuming nitric acid (HNO_3) and trifluoroacetic acid (TFA) in dichloromethane (DCM) [70]. Nonetheless, these conditions provided the corresponding octa-nitro calix[n]arene **7** only with yields lower than 20%. Fortunately, under the Dudic's conditions, which use sodium nitrate (NaNO_3) instead of fuming HNO_3 , with some modifications, we obtained **7** in 64% yield [71]. Finally, the reduction of the aromatic nitro groups of **6** and **7** to their corresponding amines was accomplished upon treatment with hydrazine hydrate in the presence of Pd/C [67,72,73]. At this point, calix[n]arenes **8** and **9** were synthe-

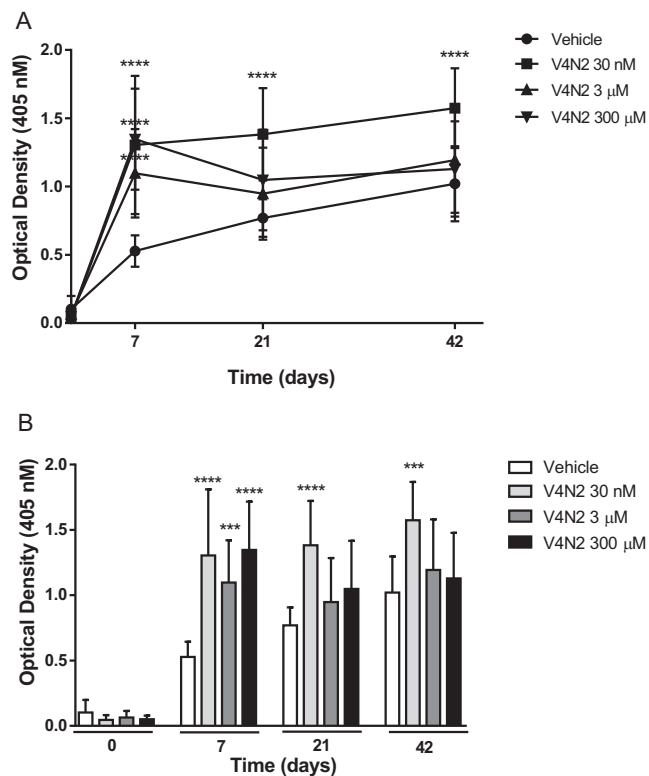


Fig. 1. (A) Effect induced by compound V4N2 (30 nM, 3 μM or 300 μM, i.p., day 0, day 7 and day 21) on the production of IgG-type anti-cocaine antibodies. Results are expressed as the time course of the experiment or (B) considering the analysis of each time, respectively. *** and **** Significantly different from vehicle ($p < 0.001$ and $p < 0.0001$, respectively). $n = 12$. Data are representative of two independent experiments.

sized from *p*-*tert*-butylphenol (**1**) in 31% and 22% yields, respectively (Scheme 1).

Next, our efforts were directed to obtain the hapten GNE. Initially, we synthesized benzoylcocaine (**13**; Scheme 2 - panels A and B), the corresponding carboxylic acid of cocaine, and amine **11** (Scheme 2 - panel A). Amine **11** was prepared in 98% yield by the protection of the commercially available 6-aminohexanoic acid (**10**) with benzyl alcohol [74], while the benzoylcocaine (**13**) was furnished in its salt form (Scheme 2 - panel B) in 84% yield from the chemoselectivity hydrolysis of the methyl ester of cocaine (**12**) [75] (Scheme 2 - panel A). Subsequently, the carboxylic acid group of **13** was coupled with amine **11**, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), furnishing amide **14** in 71% yield [44] (Scheme 2 - panel A). The benzyl ester group of **14** was then reductively cleaved by treatment with H_2 and Pd/C to provide the hapten GNE in 52% overall yield (3 steps) from cocaine (**12**) [51] (Scheme 2 - panel A).

The conjugation of GNE to calix[*n*]arenes **8** and **9** using PyBOP® as the coupling agent afforded the desired calix[*n*]arene-based immunoconjugates **V4N2** and **V8N2** (59% and 60% yields, respectively) (Scheme 3). The mass spectra of compounds **V4N2** and **V8N2** showed the $[M+H]^+$ ion [m/z 2,251.2278 (m/z calcd for $C_{132}H_{168}N_8O_{24}$ $[M+H]^+$: 2,251.2373) and m/z 4,494.7009 (m/z calcd for $C_{264}H_{343}N_{24}O_{40}$ $[M+H]^+$: 4,494.6128), respectively], one- and two-dimensional NMR spectra confirmed the expected structures. The structures of **V4N2** and **V8N2** were also validated by IR (see Supporting information for reference).

In addition to the calix[*n*]arene-based immunoconjugates **V4N2** and **V8N2**, we also prepared the immunoconjugate hapten KLH-GNE, which presents a high hydrolytic stability profile and is recog-

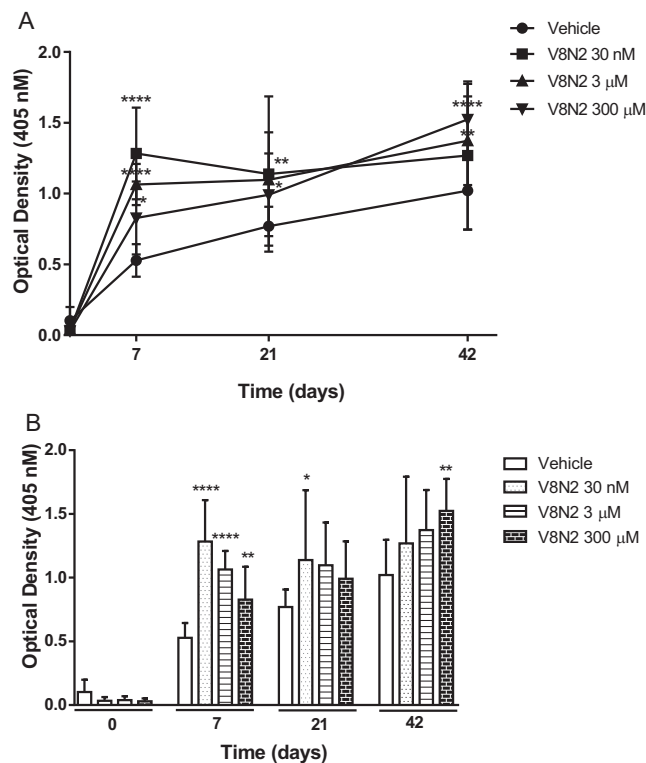


Fig. 2. (A) Effect induced by compound V8N2 (30 nM, 3 μM or 300 μM, i.p., day 0, day 7 and day 21) on the production of IgG-type anti-cocaine antibodies. Results are expressed as the time course of the experiment or (B) considering the analysis of each time (B), respectively. *, ** and **** Significantly different from vehicle ($p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively). $n = 12$. Data are representative of two independent experiments.

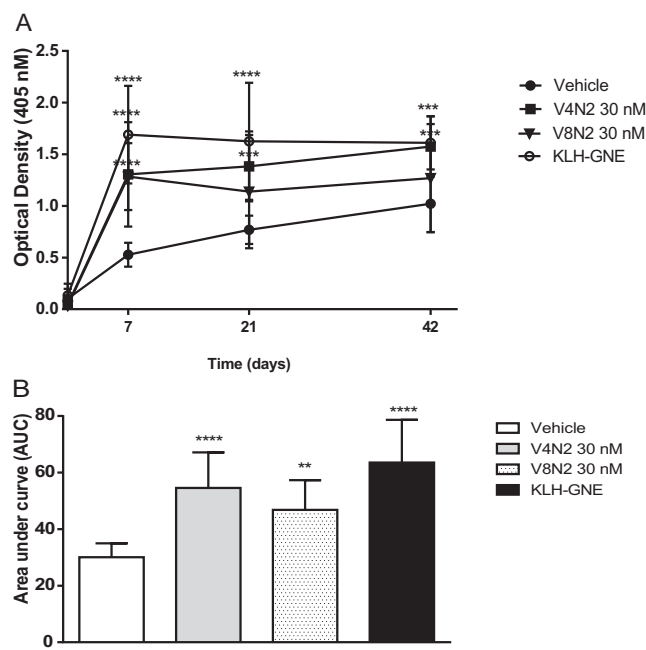


Fig. 3. (A) Effect induced by compound V4N2 (30 nM, i.p., day 0, day 7 and day 21), V8N2 (30 nM, i.p., day 0, day 7 and day 21) or KLH-GNE (positive control) on the production of IgG-type anti-cocaine antibodies. Results are expressed as the time course of the experiment or (B) the area under the curve (AUC), respectively. **, *** and **** Significantly different from vehicle ($p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively). $n = 12$. Data are representative of two independent experiments.

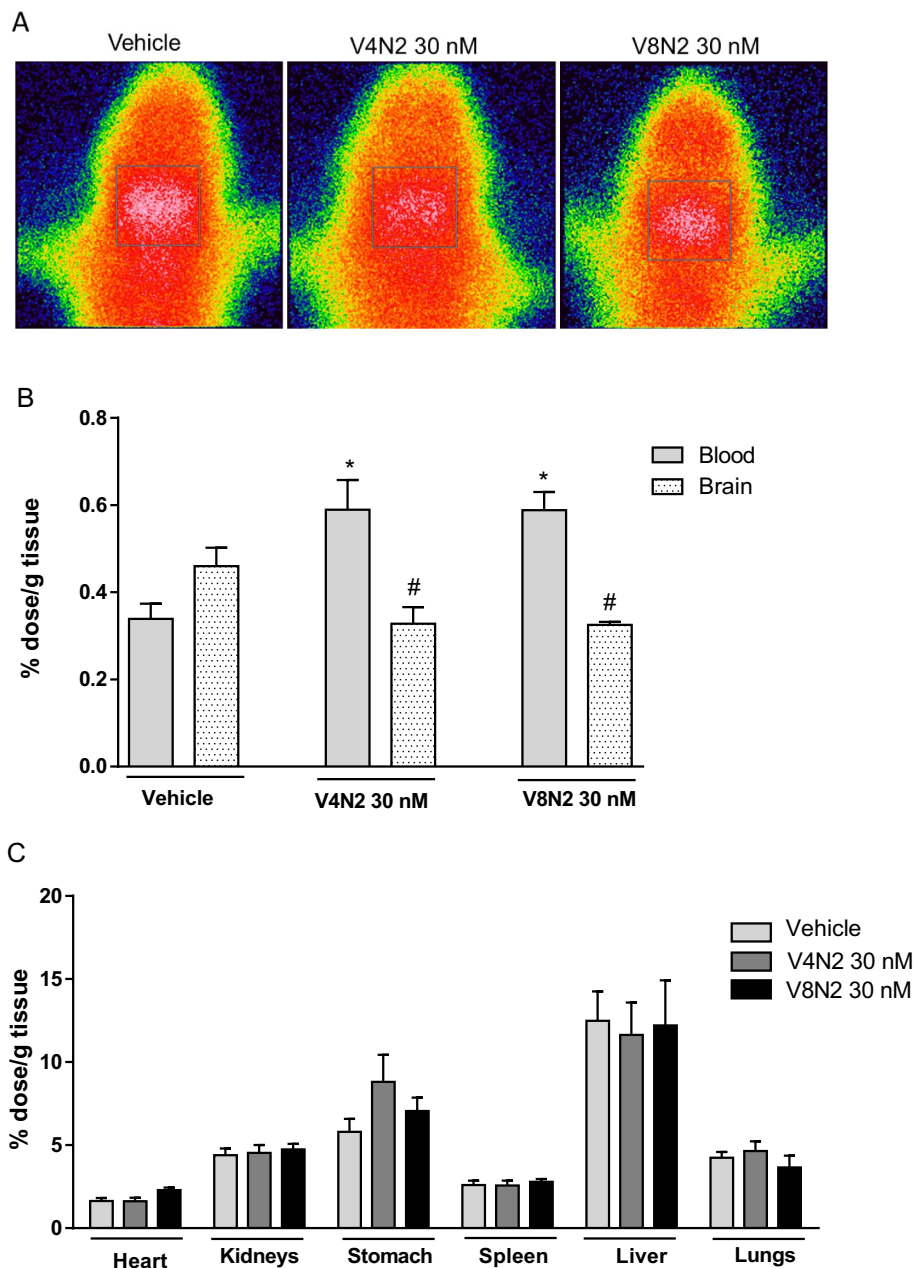


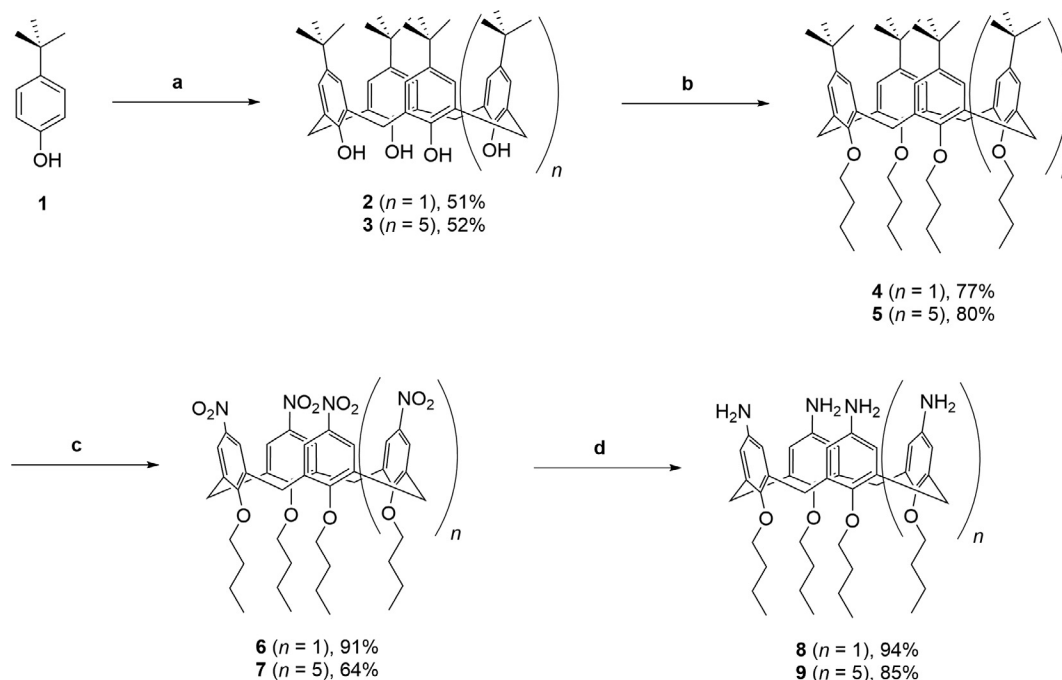
Fig. 4. Distribution of [^{99m}Tc]TRODAT-1 (+90 min, i.v) in animals previously immunized with vehicle, V4N2 or V8N2 compound (30 nM, i.p., day 0, day 7 and day 21). (A) shows the brain scintigraphy. High levels of radioactivity are represented by the light pink color. (B) Shows the distribution between the blood and the brain. * Significantly different from the vehicle in the blood ($p < 0.05$). # Significantly different from the vehicle in the brain ($p < 0.05$). (C) Shows the distribution in different organs. There were no statistically significant differences between vehicle and V4N2 (30 nM) or V8N2 (30 nM) of each organ. $n = 7$.

nized to display a potent eliciting titers of anti-cocaine antibodies with high affinity and specificity for cocaine [51]. Immunoconjugate hapten KLH-GNE was prepared following the methodology described by Cai et al. (2013) [51] and, herein, used as the positive control in our preclinical studies.

The efficacy of calix[n]arene-based immunoconjugates **V4N2** and **V8N2** at three concentrations (30 nM; 3 μM and 300 μM) was evaluated by ELISA to assess anti-cocaine antibody titers. The effect of both immunogens was also evaluated on the *in vivo* biodistribution by radiochemical studies using [^{99m}Tc]TRODAT-1, a radiolabeled analogue of cocaine. [^{99m}Tc]TRODAT-1 was employed because, similarly to cocaine, it also acts on presynaptic dopaminergic transporters and is able to cross the blood-brain

barrier, maintaining a pharmacokinetic profile similar to the one expected for cocaine [76].

Compound **V4N2** administered on days 0, 7 and 21 at the dose of 30 nM induced the production of IgG-type anti-cocaine antibodies along the whole experimental period (Fig. 1A). On the other hand, the effect produced by the doses of 3 μM or 300 μM was observed only on day 7 (Fig. 1B). Moreover, the two highest doses (3 μM and 300 μM) did not display a dose–response effect. This apparent lack of dose–response can partially be explained by the high viscosity of the formulation, which could induce a slow molecule dispersion and activation of the immune response until the day 42. Thus, in order to evaluate the distribution of [^{99m}Tc]TRODAT-1 (Fig. 4) in animals previously immunized with **V4N2**,



Scheme 1. Syntheses of calix[n]arenes 8 and 9. Reagents and reaction conditions: (a) $n = 1$, (i) NaOH, 120 °C, 2 h, (ii) DPE, 120 °C, 1 h, (iii) 260 °C, 3 h (51%) or $n = 5$, NaOH, 114 °C, 4 h, xylene (52%); (b) $n = 1$, $^n\text{BuBr}$, NaH, DMF, 60 °C, 24 h (77%) or $n = 5$, $^n\text{BuBr}$, NaH, KI, DMF, 40 °C, 72 h (80%); (c) $n = 1$, HNO_3 , TFA, DCM, 1 h, rt (91%) or $n = 5$, NaNO_3 , TFA, 7 h, rt (64%); (d) $n = 1$, $\text{H}_2\text{N-NH}_2\cdot\text{H}_2\text{O}$, Pd/C (10%), EtOH, reflux, 48 h (94%) or $n = 5$, $\text{H}_2\text{N-NH}_2\cdot\text{H}_2\text{O}$, Pd/C (10%), EtOH/THF, reflux, 24 h (86%).

we adopted the lower dose (30 nM) that exhibited activity around day 42.

Compound **V8N2** administered on days 0, 7 and 21 at the doses of 30 nM, 3 μM or 300 μM induced the production of IgG-type anti-cocaine antibodies at different times (Fig. 2A). On day 7, all the doses (30 nM, 3 μM or 300 μM) demonstrated an immune response. However, on day 21, only the lowest dose (30 nM) showed activity significantly higher compared to the vehicle group control. Finally, the highest dose (300 μM) induced the high levels of IgG antibodies only on day 42 (Fig. 2B). The high viscosity of the formulation of the doses 3 μM and 300 μM may have accounted, at least partially, for the lack of dose–response, possibly inducing a slow molecule dispersion and activation of the immune response until the last day of the evaluation. The data suggested that these doses (3 μM or 300 μM) should be evaluated over a longer period. Since the lower dose (30 nM) did not exhibit a significant difference with the highest dose (300 μM) around day 42, we selected the lower dose (30 nM) to evaluate the distribution of [$^{99\text{m}}\text{Tc}$]TRODAT-1 (Fig. 4) in animals previously immunized with substance **V8N2**.

In addition, to validate the results observed on the production of IgG-type anti-cocaine antibodies, we also investigated the effect induced by the immunoconjugate KLH-GNE (positive control), used as a reference for the antibody production, during all the times of the experiment. The immune response effect of the KLH-GNE was observed on days 7, 21 and 42 (Fig. 3A). Considering the area under the curve (AUC) along the whole experimental period, KLH-GNE and both compounds **V4N2** (30 nM) and **V8N2** (30 nM) exhibited immunological activity (Fig. 3B).

Brain scintigraphy (Fig. 4A) using [$^{99\text{m}}\text{Tc}$]TRODAT in animals previously immunized with the vehicle, **V4N2** (30 nM) or **V8N2** (30 nM) showed higher levels of radioactivity in the vehicle control group compared to the other groups (**V4N2** or **V8N2**). High levels of [$^{99\text{m}}\text{Tc}$]TRODAT-1 were observed in the blood for the treatments with **V4N2** or **V8N2**, but, at the same time, a reduced concentration of the radiotracer was noticed in the brain. On the other hand, the

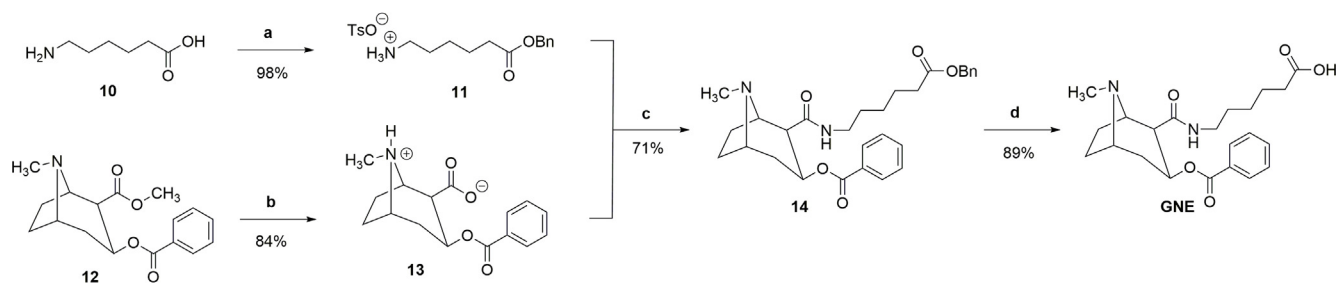
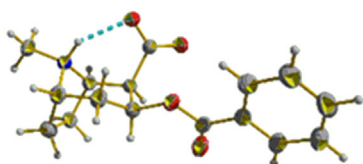
vehicle group control exhibited higher [$^{99\text{m}}\text{Tc}$]TRODAT-1 levels in the brain compared to other groups (**V4N2** or **V8N2**; Fig. 4B). The distribution of [$^{99\text{m}}\text{Tc}$]TRODAT-1 in different organs also showed that immunization with **V4N2** or **V8N2** did not influence the presence of this radiotracer in a specific organ. No differences between the groups (**V4N2** or **V8N2**) compared to vehicle group control of each organ were observed; however, for the same group it was observed a high level of [$^{99\text{m}}\text{Tc}$]TRODAT-1 in the liver compared with the other organs. The high level in the liver is expected since this organ plays a major role in xenobiotic metabolism and elevated levels of different exogenous compounds are located in this organ (Fig. 4C). Thus, the reduction of [$^{99\text{m}}\text{Tc}$]TRODAT-1 uptake in the brain, in animals previously immunized with **V4N2** or **V8N2**, can indicate that the antibodies induced by **V4N2** or **V8N2** were able to bind to [$^{99\text{m}}\text{Tc}$]TRODAT-1 in the bloodstream (Fig. 4A and B).

Conclusions

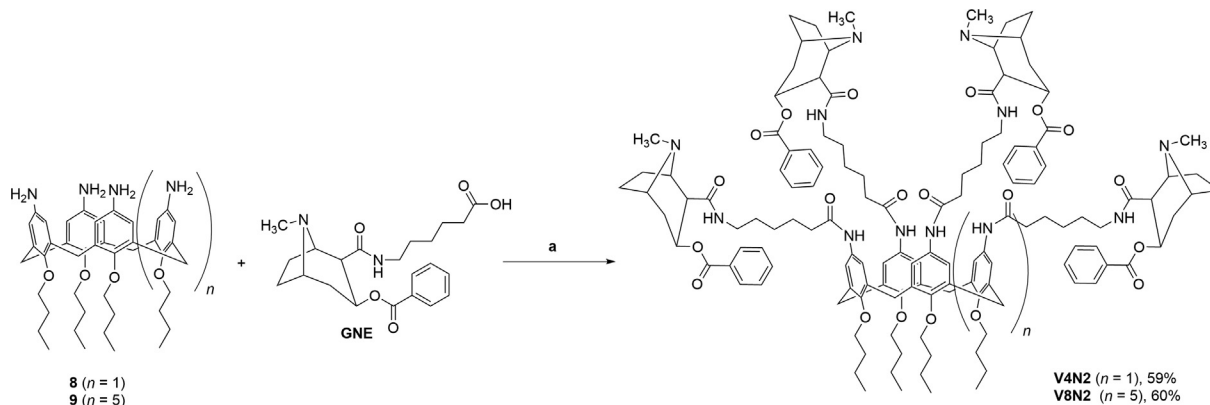
In summary, we reported the first total synthesis of the novel calix[n]arene-based immunogens **V4N2** and **V8N2** by the tethering of hydrolysis-tolerant hapten GNE on calix[4]arene and calix[8]arene moieties. Both calix[n]arene-based immunogens promoted the production of cocaine antibodies and also modulated the biodistribution of [$^{99\text{m}}\text{Tc}$]TRODAT-1, a radiolabeled analogue of cocaine. These results highlights the immunological potential of **V4N2** and/or **V8N2** as an additional strategy for the development of an immunogenic agent for the treatment of CUD.

Compliance with ethics requirements

The Local Ethics Committee in Animal Experimentation approved the study protocol (CEUA-UFGM Protocol N°. 122/2016) and at all stages of the work, the recommendations of this commission were adopted.

A:**B (CCDC: 1995677):**

Scheme 2. (A) Synthesis of cocaine hapten GNE and (B) the ORTEP diagram of 13 with 30% probability ellipsoids. Reagents and reaction conditions: (a) BnOH, PTSA, toluene, 140 °C, 24 h (98%); (b) H₂O, reflux, 24 h (84%); (c) DMAP, Et₃N, EDC, DMC, rt, 96 h (71%); (d) H₂ (40 kgf cm⁻²), Pd/C (10%), AcOEt, rt, 96 h (89%).



Scheme 3. Conjugation of hapten GNE to immunoconjugates V4N2 and V8N2. Reagents and reaction conditions: (a) $n = 1$, PyBOP, DIPEA, DCM, rt, 26 h (59%) or $n = 5$, PyBOP, DIPEA, DCM, rt, 48 h (60%).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jare.2021.09.003>.

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