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Stereoselective enzymatic synthesis of monoglucosyl-*myo*-inositols with in vivo anti-inflammatory activity

Alfonso Miranda-Molina [a], Agustín López-Munguía [b], María Luisa San Román [a], Jaime Escalante [a], Marco Antonio Leyva [c], Ana María Puebla [d], Edmundo Castillo [b,*], Laura Álvarez [a,*]

[a] *Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Morelos. Av. Universidad 1001, Col. Chamilpa, C.P. 62209, Cuernavaca-Mor., Mexico*
[b] *Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Col. Chamilpa, C.P. 62209, Cuernavaca-Mor., Mexico*
[c] *Centro De Investigación y Estudios Avanzados, Instituto Politécnico Nacional, Av. Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, C.P. 07000, México D.F., Mexico*
[d] *Centro Universitario de Ciencias Exactas e Ingeniería, Departamento de Farmacobiología, Universidad de Guadalajara, Blvd. Marcelino García Barragán 1421, esq. Calzada Olímpica, 44430, Guadalajarara, Jalisco, Mexico*

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ABSTRACT

The monoglucosyl-inositols α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol **3** and α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol **4** were synthesized by a combined enzymatic transglucosylation and hydrolysis strategy, using cyclodextrin glucosyl transferase (CGTase) from *Thermoanaerobacter* sp., followed by hydrolysis with *Aspergillus niger* glucoamylase. The glucosides were separated by preparative HPLC and fully characterized by extensive 1D and 2D NMR studies. The structure of the regioisomer **4** was confirmed by X-ray crystallography of its perbenzoylated derivative **4a**. Both isomers demonstrated in vivo anti-inflammatory activity at comparative levels to corticosterone on mouse ear oedema induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and in rat hind paw oedema induced by carrageenan.

1. Introduction

Inositols, the nine isomeric forms of hexahydroxycyclohexane, are a group of small and chemically very stable polar molecules bearing versatile biological properties. Six stereoisomers, *myo*-, *scyllo*-, *neo*-, D-*chiro*-, *epi*- and *muco*-inositol, have been recognized to occur in nature, while the remaining three, L-*chiro*-, *allo*- and *cis*-inositol are unnatural synthetic isomers.[1–4] Among them, *myo*-inositol is the most widely distributed form in microorganisms, plants, as well as in mammalian cells, where it plays very important physiological roles, such as a growth factor and a precursor of phosphatidylinositol an anti-fatty liver agent.[5] Particular attention has been devoted to glycosylated forms of *myo*-inositol due to their implication in various signal transduction pathways. Examples include inositol-containing monosaccharides such as galactinol (α-D-galactopyranosyl-(1→1)-1D-*myo*-inositol) which acts as a galactosyl donor in the synthesis of glycosinolates;[6,7] mycothiol (1D-*myo*-inosityl 2-(*N*-acethylcystein)amino-2-deoxy-α-D-glucopyranoside), a functional analogue of glutathione;[8] disaccharides such as α-D-mannopyranosyl-(1→4)-α-D-glucuronopyranosyl-(1→2)-*myo*-inositol which has effects on the amino acid metabolism of plants;[9] and more complex *myo*-inositol phospho-glycans (IPG), which have been demonstrated in numerous in vitro studies to exert partial insulin-mimetic activity on glucose and lipid metabolism in insulin-sensitive cells (adipocytes, cardiomyocytes and diaphragms).[10–12] Recently, two series of new *myo*-inositol-derived glycolipid analogues, named lanceolitols A_1–A_7 and B_1–B_7, were isolated from the leaves of the Mexican medicinal plant *Solanum lanceolatum*. These compounds showed important in vivo anti-inflammatory activity, through inhibition of phospholipase A2 and cyclooxygenase-2 (COX-2), as demonstrated by in vitro experiments.[13]

The physiological roles of *myo*-inositol and its glycosylated derivatives, as well as their potential pharmaceutical applications have focused interest in the regio- and stereoselective syntheses of these compounds. Consequently, the synthesis of partially protected inositols with free hydroxyl groups at specific positions has been the subject of intensive research.[14–20] However, due to the similar reactivity of the multiple inositol hydroxyl groups, the controlled glycosylation remains a challenge in organic chemistry.[2] In this context, considering that one of the most interesting properties of enzymes is their regio- and stereoselectivity, a biocatalytic approach represents an interesting alternative to cope with this limitation. In particular, the regio-selective glycosylation of a wide variety of chemical compounds has been achieved by the proper application of enzymes such as glycosyltransferases and glycosidases using several glycosyl donors through different

* Corresponding authors. Tel./fax: +52 777 3 29 79 97 (L.A.); tel.: +52 55 56 22 76 09; fax: +52 777 17 23 88 (E.C.).
E-mail addresses: edmundo@ibt.unam.mx (E. Castillo), lalvarez@uaem.mx (L. Álvarez).

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glycosylation strategies.[21–31] More specifically, the glycosylation of various inositols has been reported. The first successful enzymatic galactosylation of 1D-*chiro*-inositol and 1D-pinitol was achieved using a β-galactosidase from *Bacillus circulans* resulting in the synthesis of mono- and digalactosylated inositols in yields of up to 45%.[32] In a later report, the formation of 5-*O*- and 1-*O*-β-D-glucopyranosyl-*myo*-inositols and 5-*O*-β-D-galactopyranosyl-*myo*-inositol using growing cultures and enzyme extracts of *Sporobolomyces singularis* was described.[33] On the other hand, a wide variety of inositols such as 1D-*chiro*-inositol, 1D-pinitol, 1D-3-*O*-allyl-4-*O*-methyl-*chiro*-inositol, 1D-3,4-di-*O*-methyl-*chiro*-inositol, 1L-*chiro*-inositol and *myo*-inositol were successfully galactosylated with a thermophilic β-galactosidase isolated from *Thermoanaerobacter sp*. strain TP6-B1 using *p*-nitrophenyl β-D-galactopyranoside as donor, with yields ranging from 46% to 64%.[34] Inositol glucosylation has also been achieved with enzymes other than glycosidases; this is the case of kojibiose phosphorylase which was used as a biocatalyst for the synthesis of four glucosyl-*myo*-inositols.[35]

A successful approach to the synthesis of glucosylated *myo*-inositols was also reported by Sato et al., who obtained two monoglucosyl *myo*-inositols trough a transglucosylation reaction with CGTase from *Bacillus obhensis*.[35] The monoglycosylated products were effective when applied as a prebiotic on intestinal *Bifidobacterium*.[5]

CGTase (EC 2.4.1.19) is an important industrial enzyme which catalyzes the transformation of starch and linear maltodextrin substrates to produce cyclodextrins (CDs) through intramolecular transglycosylation. CDs are cyclic ring structures consisting of 6, 7 or 8 glucose residues joined by α-(1→4) linkages, designated as α-, β- and γ-CDs, respectively.[36,37] CGTases use the non-reducing end of a bound oligosaccharide as an acceptor molecule for cyclic α-(1→4)-linked oligosaccharide formation.[38] Additionally, these enzymes also catalyze three intermolecular transglycosylations: hydrolysis of starch to produce linear dextrins, a coupling reaction between cyclodextrins and/or linear dextrin to produce glucosides, and disproportionation, a type of activity in which linear oligosaccharides are recombined through transglucosylation modifying their size.[39]

Acceptors that have been successfully subjected to glycosylation by CGTases include sugar alcohols such as sorbitol;[40] sugars, including glucose, xylose, sorbose, sucrose, maltose, lactose;[27,38] glycosides such as rutin, stevioside, rebaudioside A, capsaicin, 8-nordihydrocapsaicin, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, phenyl-α-D-glucopyranoside, phenyl-β-D-glucopyranoside, sucrose laurate and alkyl glycosides; flavonoides such as hesperidin, neohesperidin, naringin[41] and ascorbic acid.[42]

The aim of the present work was to synthesize glucosyl *myo*-inositol analogues of the lanceolitols[13] through an enzymatic regio and stereoselective approach and to explore their anti-inflammatory properties. We report in detail the synthesis, purification and characterization of two monoglucosyl-inositols obtained through a glucosylation strategy using a β-cyclodextrin as a glucosyl donor and CGTase from *Thermoanaerobacter* sp. as biocatalyst, followed by the selective hydrolysis of the poly-glucosylated products with *Aspergillus niger* glucoamylase. The in vivo anti-inflammatory properties of the purified monoglucosyl-inositols are also reported.

2. Results and discussion

2.1. Enzymatic synthesis of glycosylated *myo*-inositol

Initially, the screening of various enzymes capable of performing the glycosylation of *myo*-inositol in the presence of different glycosyl donors was carried out. In all cases, the reaction products were analyzed by TLC and/or HPLC in a search for glycosylated *myo*-inositols (data not shown).

From these experiments, it became clear that *myo*-inositol is not easily recognized as an acceptor by most glycosidases, and although a large range of reaction conditions was explored; only hydrolysis and transglycosylation products (oligosaccharides) were obtained from enzymes such as α-amylase from *Aspergillus oryzae*, levansucrase from *Bacillus subtillis* and β-galactosidases from *Escherichia coli*, *A. oryzae* and *Kluyveromyces lactis*. Nevertheless, glycosylated *myo*-inositol products were observed with *Thermotoga maritima* β-glucosidase and *Thermoanaerobacter* sp. CGTase. Figure 1 shows the TLC analysis of the reaction products obtained when *T. maritima* β-glucosidase (1a) and *Thermoanaerobacter* sp. CGTase (1b) were employed. In both cases, the reaction products were compared to controls obtained with the enzyme and the glycosyl donor in the absence of *myo*-inositol (lanes 1a–7a and lanes 1c–3c). It is interesting to observe that in reactions carried out in the presence of *myo*-inositol, a series of products corresponding to mono- and/or poly-glycosylated *myo*-inositols were formed (highlighted spots in Fig. 1). In order to reduce the number of poly-glycosylated *myo*-inositol products, a glucoamylase digestion treatment of the reaction products obtained with CGTase was carried out (Scheme 1). It was found that this enzyme is capable not only of transforming the residual dextrins to glucose, through the hydrolysis of glucose units linked in the non-reducing end of the chain, but also to hydrolyze the poly-glucosylated *myo*-inositol forms to the simplest monoglucosylated forms. The reaction products obtained after glucoamylase digestion were analyzed by analytical HPLC and purified by preparative HPLC.

The modification of CGTase specificity by the presence of *myo*-inositol in the enzymatic reaction is illustrated in Figure 2, where in HPLC chromatograms, the products of CGTase activity when β-cyclodextrin is used as a substrate (Fig. 2a) are compared to those obtained in the same reaction conditions but in the presence of *myo*-inositol as acceptor (Fig. 2b). Finally, the glycosylated *myo*-inositols **3** and **4** and glucose **1**, obtained after glucoamylase digestion of the reaction products (both glycosylated *myo*-inositols as well as the maltooligosaccharides) are shown in Figure 2c.

From this analysis, the profile of transfer products obtained after 24 h of reaction with CGTase and *myo*-inositol before glucoamylase digestion were identified as: G1-*myo*-inositols **3** and **4**, G2-*myo*-inositols **5** and **6**, G3- *myo*-inositols **7** and **8** and G4-*myo*-inositol **9**, which correspond to one or several glucose molecules transferred to *myo*-inositol.[35,5] After quantification of the products it was found that 59.8% of the initial *myo*-inositol was glucosylated; 32% yield of the products were found as G1-*myo*-inositols **3** and **4** in a 73:27 ratio corresponding to 308 mM and 114 mM, respectively, 18.1% yield as G2-*myo*-inositols, 7.6% yield as G3-*myo*-inositols and 2.2% yield as G4-*myo*-inositol. After ^{1}H NMR analysis, compounds **3** and **4** were confirmed as monoglycosylated *myo*-inositol products.

These results confirm that among all the enzymatic reactions tested the combined enzymatic transglucosylation using CGTase from *Thermoanaerobacter* sp. followed by *A. niger* glucoamylase digestion result in the synthesis of monoglucosyl-*myo*-inositols.

2.2. Structural elucidation of the monoglycosylated *myo*-inositols 3 and 4

It has been reported that biological properties of glycosylated molecules rely not only on the number, type, sequence and glycosidic linkage of the glycosyl moieties, but also on the absolute configuration of the oligosaccharide.[42] Therefore, the detailed elucidation of the structural features of the oligosaccharide results are essential to fully understand their biological properties. Hence, complete structure elucidation of the monoglucosyl-*myo*-inositols **3** and **4**

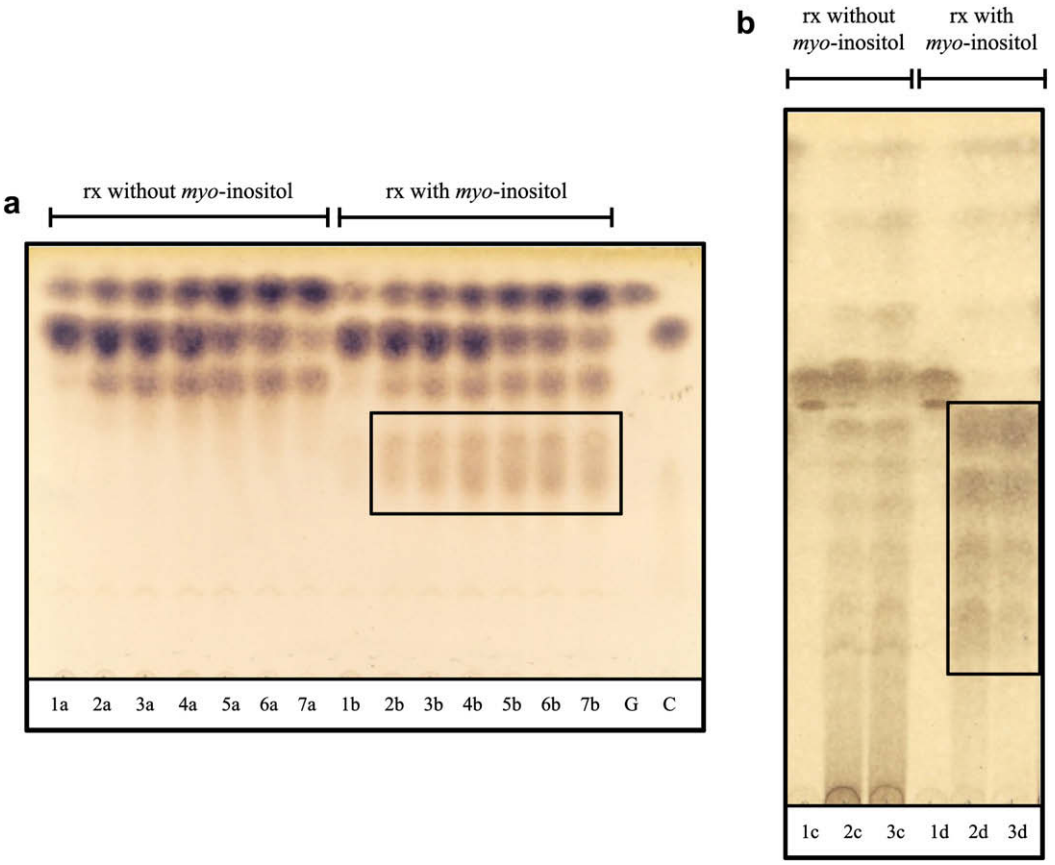
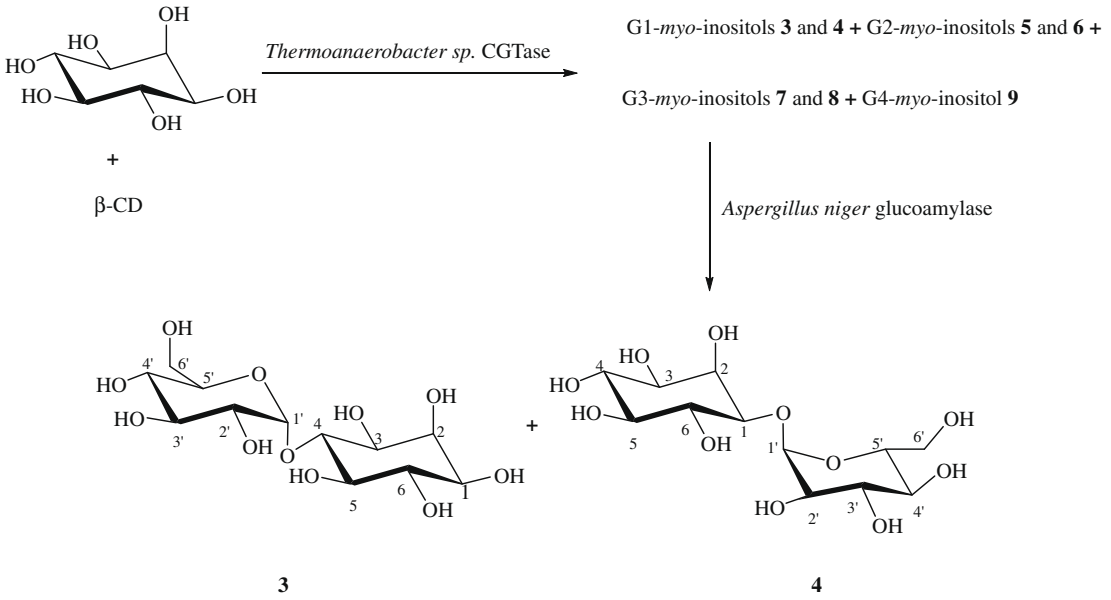


Figure 1. (a) Thin-layer chromatogram of the reaction products from cellobiose (donor) and *myo*-inositol (acceptor). Lines 1a and 1b show profiles before adding β-glucosidase; 2a–7a and 2b–7b profiles at different reaction times; G, glucose, C, cellobiose. (b) Thin-layer chromatogram of the reaction products from β-CD (donor) and *myo*-inositol (acceptor). Lines 1c and 1d show profiles before adding CGTase; lines 2c and 2d show profiles after adding CGTase at 24 h of reaction and lines 3c and 3d show profiles after 48 h of reaction.



Scheme 1. Synthetic strategy using *myo*-inositol, β-cyclodextrin as a glucosyl donor and CGTase from *Thermoanaerobacter* sp., followed by the selective hydrolysis of the poly-glucosylated products with *Aspergillus niger* glucoamylase as biocatalysts.

was determined by an extensive nuclear magnetic resonance (NMR) study. Indeed, full proton and carbon assignments were carried out using 1D (^{1}H, ^{13}C) and 2D (gCOSY, TOCSY, HETCOR, NOESY and gHMBC) NMR experiments and HRFAB mass spectrometry analysis.

HRFAB mass spectrometry analysis of compounds **3** and **4** gave a peak at *m/z* 343.1249 for $[M+H]^{+}$ corresponding to the molecular formula $C_{12}H_{23}O_{11}$. The ^{13}C NMR spectrum of the more polar compound **4** (R_t = 14.59 min) contained 12 signals. In the ^{1}H NMR spec-

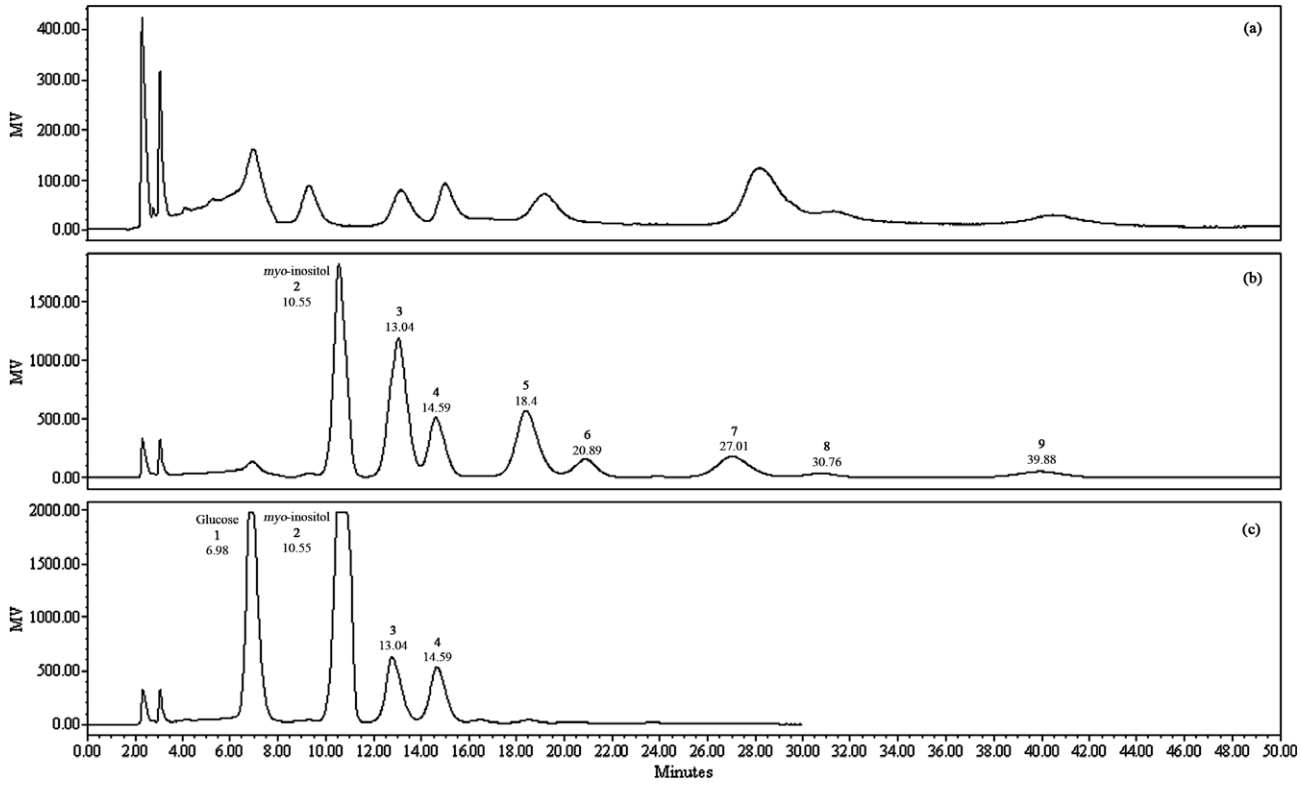


Figure 2. (a) HPLC profile of a carbohydrate mixture after 24 h of reaction with CGTase using β-cyclodextrin (β-CD) in the absence of *myo*-inositol (b) HPLC profile of a carbohydrate mixture obtained by the transglycosylation of CGTase using β-cyclodextrin (β-CD) after 24 h of reaction in presence of *myo*-inositol. (c) HPLC profile of a carbohydrate mixture obtained by glucoamylase digestion of the mixture of oligoglucosyl *myo*-inositols after 24 h. Retention times (min) are shown at the top of each peak.

trum, only one anomeric proton was observed at δ 5.15 (1H, d, J = 4.4 Hz) and HETCOR showed its attachment to the anomeric carbon at δ 98.0. This served as the starting point for tracing connectivities within the sugar spin system by gCOSY and TOCSY experiments. Once the ^{1}H signals of the glucopyranose residue have been assigned, the six remaining ^{1}H resonances were assigned to the *myo*-inositol ring, and connectivities were delineated from ^{1}H–^{1}H COSY and TOCSY experiments with the characteristic signal of H-2 at δ 4.19 (1H, dd, $J_{2,1}$ = 2.0; $J_{2,3}$ = 2.4 Hz) as the starting point. The carbon to which each hydrogen was attached was defined from HETCOR. The most downfield of these carbons (δ 79.86) in the cyclitol ring was the point of linkage to the glucose. Coupling constants ($J_{1,2}$ = 2.4 Hz; $J_{1,6}$ = 10.0 Hz) observed for its hydrogen (δ 3.57, H-1) and those observed for the hydrogen assigned to H-2 at δ 4.19 and H-3 at δ 3.50 ($J_{3,2}$ = 2.4 Hz; $J_{3,4}$ = 10.0 Hz) confirmed assignment of H-1 as an equatorial hydrogen with adjacent axial and equatorial hydrogens. The remaining assignments for the *myo*-inositol ring were straightforward. Full assignments of the proton and carbon resonances were secured from the TOCSY, COSY, NOESY and gHMBC data. Based on the gHMBC correlations, the glucose moiety was linked to C-1 of the cyclitol ring on the basis of long-range correlation between C-1 (δ 79.86) of the *myo*-inositol and H-1′ of glucose at δ 5.15. Similar analysis by NOESY established that the glucose residue was α-linked to *O*-1 of *myo*-inositol because of the correlation between H-1′ of glucose at δ 5.15 and H-1 of the cyclitol at δ 3.57.

It should be noted that *myo*-inositol is a *meso*-isomer with five equatorial hydroxyl groups and an axial hydroxyl group. The carbon bearing the axial hydroxyl group is designated as C-2, and the other ring carbons can be numbered from C-1 to C-6, starting from a C-1 atom and proceeding around the ring in clockwise or counterclockwise fashion. According to convention, a counterclockwise numbering in an asymmetrically substituted *myo*-inositol leads to the configurational D-prefix, and the clockwise numbering gives the substituted *myo*-inositol an L-prefix.[2,43,44] Therefore, substitution either side of the plane of symmetry (which bisects C-2 and C-5), such as at C-1 or C-3, results in two different compounds.

The absolute configuration of the *myo*-inositol ring was determined by an X-ray crystallographic study of the nonabenzoyl derivative **4a**. Figure 3a shows a perspective drawing of the X-ray structure of **4a**, and Figure 3b shows another perspective drawing in which, in order to gain clarity, the benzoyl groups were omitted. In this last figure it is observed the 1D-configuration of the *myo*-inositol moiety in **4a**. Therefore, compound **4** can be formulated as α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol.

This compound was obtained by a transglucosylation reaction of Kojibiose phosphorylase using *myo*-inositol as an acceptor and β-D-glucose 1-phosphate as a donor.[28] However, the ^{13}C NMR data of compound **4** obtained in this work, were very different with those described, and unfortunately, the physical data of the saccharide were not described, and we were not able to find a structural relationship between them.

A detailed analysis of the unassigned NMR correlations in the 2D NMR spectra revealed that the second product **3** was α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol, and comparison with compound **4** showed that they differ only in the linkage site of the glucose to the *myo*-inositol ring. Instead, compound **3** is attached to C-4. The presence of the glucopyranosyl unit was deduced from the ^{1}H NMR anomeric proton signal at δ 5.10 (d, J = 4.0 Hz), which was compatible with the anomeric carbon signal at δ 99.62.

Also, in this case, the proton-coupling network within the pseudo-disaccharide residue was established using a combination of ^{1}H–^{1}H gCOSY, NOESY, TOCSY, HETCOR and gHMBC experiments. Based on the gHMBC correlations, the glucose moiety was linked to C-4 of the cyclitol ring on the basis of long-range correlation be-

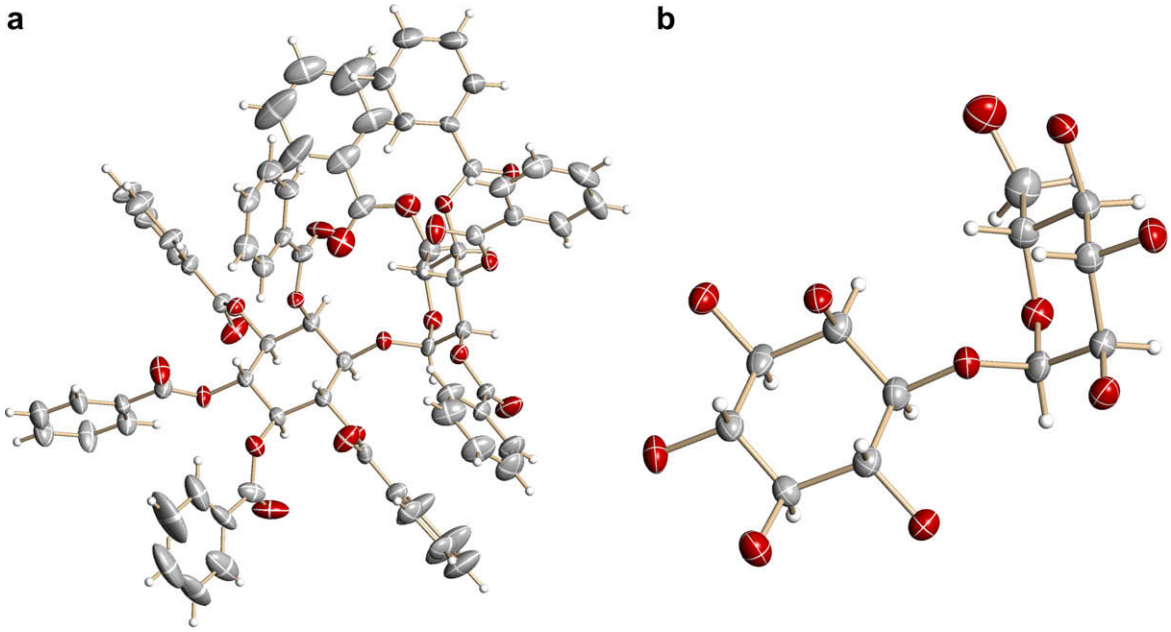


Figure 3. (a) A stereoscopic view of compound **4a**. (b) Perspective drawing in which, in order to gain clarity, the benzoyl groups were omitted.

tween C-4 (δ 80.92) of *myo*-inositol and the H-1′ of glucose moiety at δ 5.10. Moreover, NOESY experiment established that the glucose residue was α-linked to *O*-4 of *myo*-inositol because a correlation between H-1′ of glucose at δ 5.10 and H-4′ of the cyclitol at δ 3.52 was observed. This compound was previously obtained by Sato et al.[35] by using a transglucosylation reaction of CGTase from *B. obhensis*, *myo*-inositol as an acceptor and β-cyclodextrin as donor. The physical and NMR data of compound **3** and its nanobenzoyl derivative **3a** were very similar with those described,[35,45] indicating that these compounds have the same configuration. Therefore compound **3** can be formulated as α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol.

In addition, it was noteworthy the downfield shift of 0.17 ppm displayed by H-5′ in the ^{1}H NMR spectra of the regioisomer **3**, in comparison with H-5′ in compound **4**. In order to understand this downfield shift, we performed a theoretical study of the two possible diasteromeric products that could be obtained by glucosylation at the enantiotopic 4- and 6-positions of *myo*-inositol. For this, we employed PM3 calculations at the semi empiric level, with correction of the H–H interactions, with the SPARTAN programme. These calculations predicted considerably shorter interatomic distance between H-5′ and OH-C-6′ for D-*myo*-inositol (2.588 Å) than the L-isomer (2.629 Å). This result shows that the cyclitol ring has the D-configuration.

3. Biological activity

Taking into consideration that the natural lanceolitols (for example: D-*myo*-inositol-2-*O*-dodecanoyl-1-*O*-β-D-glucopyranoside), isolated previously from *S. lanceolatum*, showed important anti-inflammatory activity,[13] we carried out a preliminary study exploring the anti-inflammatory properties of the regioisomeric analogues **3** and **4**. For this purpose both compounds were tested in vivo for their ability to reduce the inflammatory response in the TPA-induced ear oedema and carrageenan-induced paw oedema of mice, and the inhibitory activity was compared with that of corticosterone (Cort), a commercially available steroidal (SAI) anti-inflammatory drug. Taking the anti-inflammatory activity after 2.5 h as criteria for comparison, it can be concluded that α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol **3** displayed a higher inhibition of ear oedema (80%) at a dose of 1.5 mg/kg of body weight, than corticosterone (52.5%) at a dose of 10 mg/kg of body weight; whereas α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol **4** was nearly equipotent (40 % of inhibition) to corticosterone at the same dose. In the carrageenan-induced oedema test, both compounds **3** and **4** showed an inhibition of paw oedema of 76% and 80%, respectively at a dose of 1.5 mg/kg, comparable to the reference drug (79%) at a dose of 10 mg/kg. The present data clearly showed that compounds **3** and **4** exhibit important anti-inflammatory activity on inhibiting oedema formation after carrageenan subplantar injection, and TPA administration in the ear of mice. Further studies must be conducted in order to clarify the exact anti-inflammatory mechanism of these compounds.

4. Conclusion

The coupling reaction between β-CD and *myo*-inositol via an enzymatic glycosylation with CGTase from *Thermoanaerobacter* sp. and selective hydrolysis of the poly-glucosylated products with glucoamylase from *A. niger*, provides a versatile method to produce monoglucosyl-*myo*-inositols in moderate yield (32%). The products obtained were identified as α-D-glucopyranosyl-(1→4)-4D-*myo*-inositol **3** and α-D-glucopyranosyl-(1→1)-1D-*myo*-inositol **4**. In addition, this study was able to demonstrate that CGTase from *Thermoanaerobacter* sp. stereoselectively glycosylate the (1*S*)- and (4*S*)-positions of *myo*-inositol.

These regioisomeric α-D-glucopyranosyl *myo*-inositols **3** and **4**, markedly inhibited inflammation induced in mice. This is the first report about the anti-inflammatory properties of α-glucosyl *myo*-inositols, which allow us to consider them as a useful tool for obtaining new analgesic and anti-inflammatory agents.

5. Experimental

5.1. General

Melting points were determined on a Fisher Johns melting point apparatus and were not corrected. HRFABMS spectra in a matrix of *m*-nitrobenzyl alcohol were recorded on a JEOL JMX-AX 505 HA mass spectrometer. Optical rotations were measured on a

Perkin–Elmer 241MC polarimeter (10 cm, 1 mL cell). The benzoylation reactions were made in a CEM microwave apparatus. X-ray crystallographic data were acquired in an Enraf–Nonius–Bruker Kappa CCD equipment; method used for solve the structure: software XM SHELXTL.[46] NMR spectra were recorded on a Varian Unity NMR Spectrometer at 400 MHz for ^{1}H NMR, ^{1}H-^{1}H gCOSY, NOESY, gHMBC, HETCOR, HSQC and ^{1}H-^{1}H TOCSY and 100 MHz for ^{13}C NMR using D_2O for compounds **3** and **4** and $CDCl_3$ for compounds **3a** and **4a** as solvent. NMR experiments are referenced to H_2O and TMS and chemical shifts are reported in ppm (δ).

T. maritime β-glucosidase was obtained using a similar procedure reported for α-amylase AmyA from the same strain.[47] CGTase from *Thermoanaerobacter* sp. (Toruzyme® 3.0 L, a liquid enzyme preparation) was obtained from Novozyme. *Myo*-inositol was obtained from Sigma. β-cyclodextrin was obtained from American Maize-Products Company. Glucoamylase from *A. niger* used for digestion of oligoglucosyl-inositols was obtained by ANZECO®.

5.2. Thin-layer chromatography

The TLC was performed on a Silica Gel 60 (Merck) pre-coated plate with a solvent system (v/v) of *n*-butanol/ethanol/water (3:5:2). The spots of sugars were detected by spraying with α-naphthol solution, followed by heating at 120 °C for 10 min.

5.3. Transglucosylation reaction with β-glucosidase from *T. maritima*

Solutions of *myo*-inositol 1.0 M and 1.0 M of cellobiose were prepared in 100 mM K_2HPO_4 buffer at pH 6.0 and β-glucosidase from *T. maritima* was added at a final concentration of 5 units/mL. The transglucosylation reaction was performed at a final volume of 3 mL, at 70 °C for 48 h. The reaction mixture was stored at −18 °C until subsequent analyzes and compared to blank experiments obtained with the enzyme in absence of *myo*-inositol.

5.4. Transglucosylation reaction with CGTase from *Thermoanaerobacter* sp.

Solutions of *myo*-inositol 1.07 M and 0.17 M of β-CD were prepared in 50 mM Na_2HPO_4 buffer at pH 6.0 and CGTase enzyme from *Thermoanaerobacter* sp. was added at a final concentration of 8.6 units/mL. The transglucosylation reaction was performed at a final volume of 3 mL, at 50 °C for 24 h. The reaction mixture was immersed in boiling water for 10 min to inactivate the enzyme and was stored at -18 °C until subsequent analyzes and compared to blank experiments obtained with the enzyme in the absence of *myo*-inositol.

5.5. Glucoamylase digestion of incubated broth and purification of monoglucosyl-*myo*-inositols

An incubated broth (3 mL) of CGTase was digested with 258 U/mL of glucoamylase at 50 °C, in buffer (pH 6.0) Na_2HPO_4 for 24 h. The reaction was stopped by boiling for 10 min and was stored at −18 °C until subsequent high-performance chromatographic separation.

Formation of oligoglucosyl-inositols and monoglucosyl-inositols was monitored by high-performance liquid chromatography (HPLC). The measurement conditions were as follows: Colum Prevail Carbohydrate ES 5μ 4.6 mm × 250 mm; eluent, CH_3CN–H_2O (68:32 v/v); flow rate, 1.0 mL/min; colum temperature, 32 °C; RI detector (Waters). The injection vol. was 10 μL.

After glucoamylase digestion treatment of the reaction products obtained with CGTase, compounds **3** and **4** were separated by preparative HPLC. The HPLC conditions were as follows: Column μBondapak™ NH_2 10 μm, 125 Å, 7.8 × 300 mm (waters); eluent CH_3CN–H_2O (80:20 v/v); flow rate, 7.0 mL/min; column temperature, 35 °C; RI detector (waters).

5.5.1. α-D-Glucopyranosyl-(1→4)-4D-*myo*-inositol 3

White amorphous powder, mp 153–155 °C (decomposed), $[\alpha]_D^{20} = +66.5$ (*c* 0.1, H_2O). ^{1}H NMR (400 MHz, D_2O): δ = 5.10 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 3.83 (dd, 1H, $J_{2,1}$ = 2.8, $J_{2,3}$ = 3.2 Hz, H-2), 3.78 (ddd, 1H, $J_{5',6a'}$ = 2.8, $J_{5',6b'}$ = 4.8, $J_{5',4'}$ = 10, H-5′), 3.61 (m, 1H, H-6a′), 3.55 (dd, 1H, $J_{6,1}$ = 8.8, $J_{6,5}$ = 10.0 Hz, H-6 or H-4), 3.54 (m, 1H, H-6b′), 3.52 (dd, 1H, $J_{4,3}$ = 9.2, $J_{4,5}$ = 10.0 Hz, H-4 or H-6), 3.42 (dd, 1H, $J_{3,2}$ = 3.2, $J_{3,4}$ = 9.8 Hz, H-1 or H3), 3.40 (dd, 1H, $J_{3',4'}$ = 9.6, $J_{3',2'}$ = 10.0 Hz, H-3′), 3.34 (dd, 1H, $J_{2',1'}$ = 4.0, $J_{2',3'}$ = 9.6 Hz, H-2′), 3.30 (dd, 1H, $J_{1,2}$ = 2.8, $J_{1,6}$ = 10.4 Hz, H-3 or H1), 3.25 (dd, 1H, $J_{5,4}$ = 8.8, $J_{5,6}$ = 10.0 Hz, H-5), 3.21 (dd, 1H, $J_{4',3'}$ = 9.2, $J_{4',5'}$ = 10.0 Hz, H-4′). ^{13}C NMR (100 MHz, D_2O): δ = 99.62, 80.92, δ 74.85, 73.21, 72.69, 72.48, 72.18, 71.99, 71.23, 69.99, 68.68, 60.70. HRFABMS (positive mode), calcd for $C_{12}H_{23}O_{11}$: 343.3759, found: 343.1249.

5.5.2. α-D-Glucopyranosyl-(1→1)-1D-myo-inositol 4

White amorphous powder, mp 155–157 °C (decomposed), $[\alpha]_D^{20} = +85.8$ (*c* 0.1, H_2O). ^{1}H NMR (400 MHz, D_2O): δ = 5.15 (d, 1H, $J_{1',2'}$ = 4.4 Hz, H-1′), 4.19 (dd, 1H, $J_{2,1}$ = 2.0, $J_{2,3}$ = 2.4 Hz, H-2), 3.82 (m, 1H, H-6′b), 3.80 (dd, 1H, $J_{6,1}$ = 9.2, $J_{6,5}$ = 10.0 Hz, H-6 or H4), 3.76 (dd, 1H, $J_{4,3}$ = 8.8, $J_{4,5}$ = 10.0 Hz, H-4 or H6), 3.75 (dd, 1H, $J_{3',2'}$ = 8.8, $J_{3',4'}$ = 10.0 Hz, H-3′), 3.72 (m, 1H, H-6′a), 3.61 (m, 1H, H-5′), 3.57 (dd, 1H, $J_{1,2}$ = 2.4, $J_{1,6}$ = 10.0 Hz, H-1or H-3), 3.52 (dd, 1H, $J_{2',1'}$ = 4.4, $J_{2',3'}$ = 10.0 Hz, H-2′), 3.50 (dd, 1H, $J_{3,2}$ = 2.4, $J_{3,4}$ = 10.0 Hz, H-3 or H-1), 3.38 (dd, 1H, $J_{4',3'}$ = 9.6, $J_{4',5'}$ = 10.0 Hz, H-4′), 3.28 (dd, 1H, $J_{5,4}$ = 9.2, $J_{5,6}$ = 10.0 Hz, H-5). ^{13}C NMR (100 MHz, D_2O): δ = 100.81, 79.86, 74.30, 73.15, 72.63, 72.42, 72.21, 72.05, 72.05, 71.39, 69.93, 60.92. HRFABMS (positive mode), calcd for $C_{12}H_{23}O_{11}$: 343.3759, found: 343.1249.

5.6. General benzoylation procedure

Benzoyl chloride was added dropwise to a 10 mL glass microwave reaction vessel at room temperature containing a stirred solution of **3** or **4** in pyridine. The reaction vessel was sealed with a cap and then placed into the microwave cavity. The microwave was programmed to heat the reaction mixture to the desired temperature. After the reaction was completed, the vessel was cooled below 50 °C using a flow of compressed air. The reaction was quenched with ice (5 mL), washed with water, 1 M hydrochloric acid, dried ($NaSO_4$), filtered and extracted with CH_2Cl_2 (3 × 25 mL) and concentrated. The product was purified by column chromatography (hexane-ethyl acetate, 90:10→50:50) on silica gel to give the corresponding perbenzoylated derivatives.

5.6.1. α-D-(2,3,4,6-Tetra-*O*-benzoyl)-glucopyranosyl-(1→4)-4D-(1,2,3,5,6-penta-*O*-benzoyl)-*myo*-inositol 3a

Prepared from **3** (0.0236 g, 0.069 mmol), benzoyl chloride (0.172 mL, 1.48 mmol) and pyridine (0.26 mL). The reaction was heated at 90 °C with 50 W and 13 psi for 30 min to afford compound **3a** (in 75 % yield) as a white solid after purification; mp 139–141 °C, $[\alpha]_D^{20} = +72.95$ (*c* 1.05, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.3–7.0 (m, 45H, ArH), 6.29 (t, 1H, $J_{2,1}$ = 2.8, $J_{2,3}$ = 2.8 Hz, H-2), 6.24 (t, 1H, $J_{6,1}$ = 10.4, $J_{6,5}$ = 10.4 Hz, H-6 or H4), 6.02 (dd, 1H, $J_{5,4}$ = 9.6, $J_{5,6}$ = 10.0 Hz, H-5), 5.98 (dd, 1H, $J_{3,2}$ = 3.2, $J_{3,4}$ = 10.0 Hz, H-3 or H-1), 5.93 (dd, 1H, $J_{3',2'}$ = 10.4, $J_{3',4'}$ = 10.0 Hz, H-3′), 5.82 (dd, 1H, $J_{1,2}$ = 3.2, $J_{1,6}$ = 10.4 Hz, H-1 or H-3), 5.73 (d, 1H, $J_{1',2'}$ = 3.6 Hz, H-1′), 5.59 (dd, 1H, $J_{4',3'}$ = 10.0, $J_{4',5'}$ = 10.4 Hz, H-4′), 5.22 (dd, 1H, $J_{2',1'}$ = 3.6, $J_{2',3'}$ = 10.4 Hz, H-2′), 5.01 (dd, 1H, $J_{4,3}$ = 9.6, $J_{4,5}$ = 10.0 Hz, H-4 or H6), 4.35 (m, 1H, H-6′a), 4.35 (m, H-5′), 3.68 (br d, H-6′b). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.14, 165.67, 165.52, 165.43, 165.36, 165.09, 164.73, 134.05, 133.74,

133.36, 133.26, 133.0, 130.12, 129.99, 129.83, 129.80, 129.68, 129.62, 129.11, 128.95, 128.66, 128.48, 128.40, 128.29, 128.19, 128.14, 96.81, 73.83, 73.12, 70.84, 70.63, 70.23, 70.13, 69.65, 69.49, 68.52, 68.18, 61.87. HRFABMS (positive mode), calcd for $C_{75}H_{58}O_{20}$: 1279.2510, found: 1279.3827.

5.6.2. α-D-(2,3,4,6-Tetra-*O*-benzoyl)-glucopyranosyl-(1→1)-1D-(2,3,4,5,6-penta-*O*-benzoyl)-*myo*-inositol 4a

Prepared from **4** (0.0364 g, 0.12 mmol), benzoyl chloride (0.27 mL, 2.32 mmol) and pyridine (0.4 mL). The reaction was heated at 90 °C with 50 W and 13 psi for 30 min, to afford compound **4a** (77.85% yield) as a white solid after purification, crystallized from acetone/methanol (1:3), mp 115–118 °C, $[\alpha]_D^{20} = +30.5$ (*c* 2.58, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ = 8.5–7.0 (m, 45H, ArH), 6.36 (dd, 1H, $J_{2,1}$ = 2.8, $J_{2,3}$ = 3.2 Hz, H-2), 6.32 (t, 1H, $J_{6,1}$ = 10.4, $J_{6,5}$ = 10.4 Hz, H-6 or H4), 6.22 (dd, 1H, $J_{4,3}$ = 10.0, $J_{4,5}$ = 10.4 Hz, H-4 or H6), 5.97 (dd, 1H, $J_{3',2'}$ = 9.6, $J_{3',4'}$ = 10.0 Hz, H-3′), 5.82 (dd, 1H, $J_{5,4}$ = 10.0, $J_{5,6}$ = 10.4 Hz, H-5), 5.73 (dd, 1H, $J_{3,2}$ = 3.2, $J_{3,4}$ = 10.6 Hz, H-3 or H-1), 5.66 (dd, 1H, $J_{4',3'}$ = 9.6, $J_{4',5'}$ = 10.0 Hz, H-4′), 5.59 (d, 1H, $J_{1',2'}$ = 4.0 Hz, H-1′), 5.22 (dd, 1H, $J_{2',1'}$ = 3.6, $J_{2',3'}$ = 10.4 Hz, H-2′), 4.78 (ddd, $J_{5',6'a}$ = 3.2, $J_{5',6'b}$ = 4.8, $J_{5',4'}$ = 8.6, H-5′), 4.67 (dd, 1H, $J_{1,2}$ = 3.2, $J_{1,6}$ = 8.4 Hz, H-1 or H-3), 4.64 (m, 1H, H-6′a), 4.54 (dd, $J_{6'b,5'}$ = 4.0, $J_{6'b,6'a}$ = 12.8, H-6b′). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.34, 166.31, 165.64, 165.55, 165.33, 165.26, 165.17, 165.12, 164.80, 133.85, 133.55, 133.39, 133.26, 133.21, 133.14, 133.09, 132.92, 130.42, 130.06, 130.01, 129.98, 129.92, 129.81, 129.62, 129.40, 129.28, 129.08, 129.0, 128.84, 128.72, 128.61, 128.51, 128.45, 128.29, 128.20, 128.02, 98.00, 74.13, 72.02, 71.31, 71.02, 70.78, 70.78, 70.20, 70.05, 69.23, 62.81. HRFABMS, calcd for $C_{75}H_{58}O_{20}$: 1279.2510, found: 1279.4065. X-ray crystallographic structure in Figure 3.[48]

5.7. Biological evaluation

5.7.1. Experimental animals

Adult male BALB/c mice with a body weight ranging from 20 to 22 g were used. All animals had free access to food and water and were kept on a 12/12 h light-dark cycle.

5.7.2. TPA-induced mouse ear oedema

Oedema was induced by topical application of 2.5 μg of TPA (12-*O*-tetradecanoylphorbol-13-acetate) dissolved in 20 μL acetone to the right ear of ten mice. Solutions of compounds **3** and **4** (1.5 mg/kg) were dissolved in 20 μL of physiological solution and were applied 30 min before TPA administration. A group of five mice were treated with the standard drug corticosterone (10 mg/kg) as reference. Left ears of five mice (control) were treated with 20 μL acetone only. The ear swelling was measured with a micrometre before and at 0.5, 1, 2 and 2.5 h after TPA application. The oedema was expressed as the increase in thickness in μm.

5.7.3. Carrageenan-induced mouse paw oedema

Oedema was induced in the right hind paw by subplantar injection of carrageenan (0.1% w/v in physiological solution, 20 μL) of five mice. Compounds **3** and **4**, dissolved in physiological solution were administered ip at a dose of 1.5 mg/kg, 30 min before carrageenan injection. A group received the reference drug corticosterone (10 mg/kg, po). The left paw volumes were measured with a micrometre at zero time prior to carrageenan and at 0.5, 1, 2 and 2.5 h after inflammation induction. The oedema was expressed as the increase in thickness.

5.7.4. Statistical analysis

Data are expressed as a mean SEM. Data were analyzed by using Dunnett's test post Anova test. *P* values <0.05 were considered to be significant.

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48. Crystallographic data (excluding structure factors) for **4a** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 737220. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44(0) 336033 or e-mail: deposit@ccdc.cm.ac.uk].