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Anacardic acid derivatives as inhibitors of glyceraldehyde-3-phosphate dehydrogenase from *Trypanosoma cruzi*

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ABSTRACT

Chagas' disease, a parasitic infection caused by the flagellate protozoan *Trypanosoma cruzi*, is a major public health problem affecting millions of individuals in Latin America. On the basis of the essential role in the life cycle of *T. cruzi*, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been considered an attractive target for the development of novel antitrypanosomatid agents. In the present work, we describe the inhibitory effects of a small library of natural and synthetic anacardic acid derivatives against the target enzyme. The most potent inhibitors, 6-*n*-pentadecyl- (**1**) and 6-*n*-dodecylsalicilic acids (**10e**), have IC_{50} values of 28 and 55 μM, respectively. The inhibition was not reversed or prevented by the addition of Triton X-100, indicating that aggregate-based inhibition did not occur. In addition, detailed mechanistic characterization of the effects of these compounds on the *T. cruzi* GAPDH-catalyzed reaction showed clear noncompetitive inhibition with respect to both substrate and cofactor.

1. Introduction

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a major cause of illness, morbidity, long-term disability, and death in Latin America.[1] Currently, there are over 9 million people infected with *T. cruzi*, resulting in a variety of adverse health events such as heart failure, with more than 50,000 deaths each year. It is thought that another 100 million people are at risk of infection. In spite of the alarming health, economic, and social consequences of this parasitic infection, the limited existing drug therapy (nifurtimox and benznidazole) suffers from a combination of drawbacks including poor efficacy, and serious side effects. Therefore, there is an urgent need for new safe and effective therapy against Chagas' disease.[2,3]

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a key enzyme involved in the parasite's glycolytic pathway. This homotetrameric enzyme catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate (1,3DPGA) in presence of NAD^+ and inorganic phosphate.[4]

GAPDH plays a central role in controlling ATP production in pathogenic parasites such as *T. cruzi*, *T. brucei*, and *Leishmania* sp., the causative agents of Chagas' disease, sleeping sickness, and leishmaniasis, respectively.[5–7] On the basis of the essential role in the life cycle of trypanosomes, GAPDH has been considered an important target for drug development. Considering that intracellular amastigotes depend on glycolysis for ATP production, the inhibition of the glycosomal GAPDH would prevent *T. cruzi* from being infective.[2,8] In addition, the enzyme GAPDH from the pathogenic parasites *T. cruzi*,[9] *T. brucei*,[10] and *Leishmania mexicana*[11] are closely related (about 90% sequence identity) and possess important structural differences for drug design when compared to the homologue protein of the mammalian host (about 45% sequence identity).

Several natural products and synthetic compounds have been evaluated against *T. cruzi* GAPDH using a standard biochemical assay.[12–15] Among these, a mixture of anacardic acids, isolated from the Brazilian cashew-nut shell liquid, presented promising results.[16] Chemically, anacardic acids feature a convenient salicylic acid system and a long side chain at the 6-position, in which a double bond is found at C-8 in the monoene, diene and triene components. These compounds exhibit a wide range of biological activities (e.g., antimicrobial, antitumoral, molluscicide, antifungal, insecticide),[17–21] stimulating the search for new derivatives with improved properties.[22–29,18] A variety of synthetic methods for the preparation of anacardic acids,[30–37] as well as for converting these materials to other useful compounds, has been reported.[37,38] As part of our research program aimed at discovering novel *T. cruzi* GAPDH inhibitors and in order to identify the mode of action of new inhibitors, we have synthesized and evaluated a small library of anacardic acid analogues.

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2. Results and discussion

In the present study, we have investigated the activity of *Anacardium occidentale* (cashew, a tree in the flowering plant family Anacardiaceae) extracts against *T. cruzi* GAPDH. Cashew nut shell liquid is a rich source of anacardic acids, and the access to these compounds was possible through the utilization of rotation locular counter-current chromatography (RLCC).[39] A mixture of anacardic acids containing different degrees of unsaturation in the alkenyl side chain was obtained, and produced about 80% GAPDH inhibition at a dose of 1 mg/mL. Next, we have reduced by hydrogenation the double bonds of the different components of the mixture, obtaining a single derivative, as shown in the Figure 1. This strategy allowed the evaluation of the pure anacardic acid derivative **1**, which has an IC_{50} value of 28 μM, and can be considered one of the most potent inhibitor of *T. cruzi* GAPDH yet described.

Based on these preliminary results, we have employed the methodology described by Yamagiwa et al.[30] in order to prepare a series of anacardic acid derivatives starting from a common intermediate (**6**) (Scheme 1). The reduction of *m*-anisaldehyde with $NaBH_4$ furnished alcohol **2** that was treated with $SOCl_2$ to give compound **3**, which when reacting with dimethylamine led to amine **4**. Lithiation of this with *n*-BuLi in THF at 0 °C, followed by quenching with a large excess of ethyl chloroformate afforded **5** that after reaction with triphenylphosphine provided the fosfonium salt **6**. The Wittig reaction was carried out in order to extend the side chain at the C-6 position; thus, the phosphonium salt **6** was treated with lithium bis(trimethylsilyl)amide (LHMDS) at −78 °C and quenched with different aldehydes to afford the products with the correspondent side chains (n = 4–9, R = OTHP/CH_3).

The next steps were reduction of the double bound using 5% palladium on carbon as catalyst to give **8a–e** and demethylation with $AlCl_3$ (**8a–c**) or BBr_3 (**8d**,**e**) to furnish **9a–e**. Finally, an alkaline hydrolysis in refluxing aqueous DMSO provided the anacardic acids analogs. Derivative **11**,[40] containing a terminal aldehyde group, was obtained through Swern oxidation of **10**. The acids were obtained in overall yields of 1.8–12.2% (6% average) from amine intermediate **4**.

Among the synthesized anacardic acids are compounds containing 6–12 carbons in the alkyl side chain, with the presence or absence of a polar group at the end, and also substituted at the carboxyl or methoxyl groups. Twenty-one derivatives were prepared (Table 1) and then submitted to biochemical evaluation against *T. cruzi* GAPDH (Table 2).

The percentage of inhibition was calculated according to the following equation:

$$\%\ \text{Inhibition} = 100 \times (1 - V_i/V_0)$$

where V_i and V_0 are the initial velocities (enzyme activities) determined in the presence and in the absence of inhibitor, respectively. Values of IC_{50} were independently determined by making rate measurements for at least five inhibitor concentrations. The IC_{50} values correspond to the concentration of compound required for 50% inhibition of GAPDH, and were determined from the collected data by nonlinear regression analysis.

As can be seen in Table 2, the two most potent inhibitors, among the 21 derivatives evaluated against the parasite enzyme,

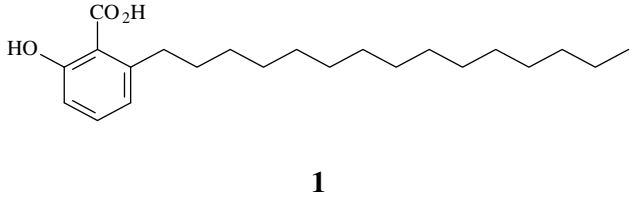

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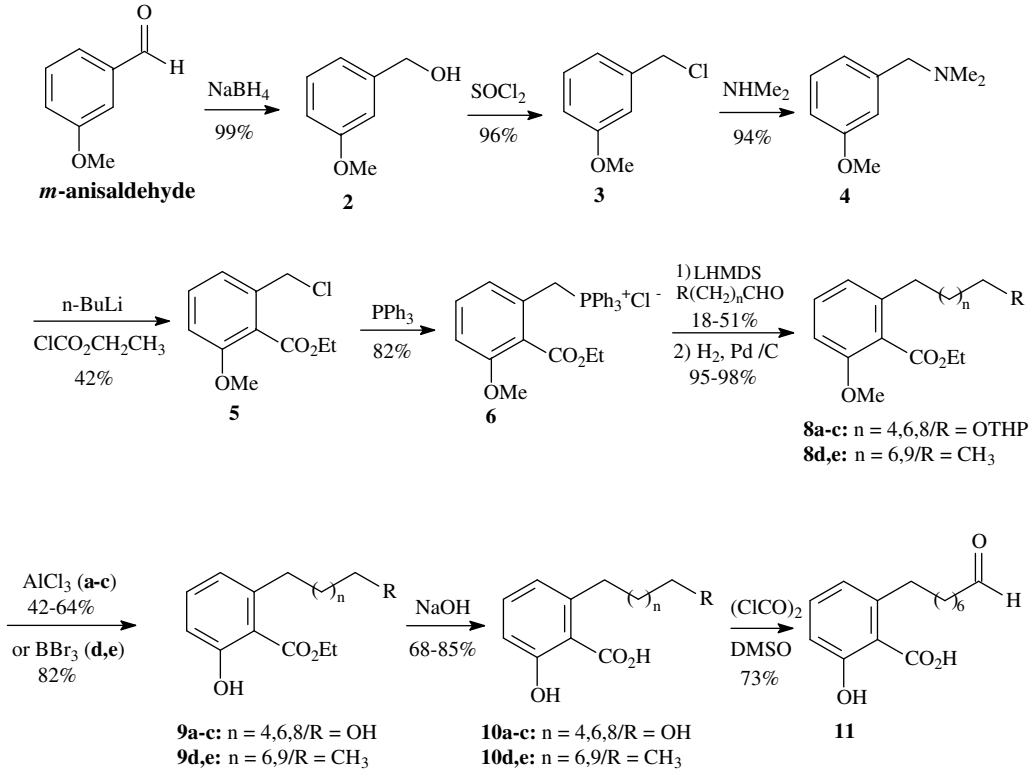
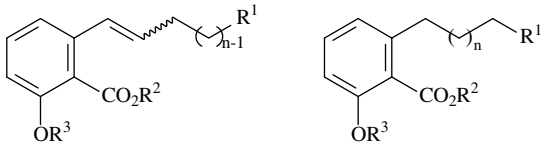
Figure 1. Chemical structure of anacardic acid derivative **1**, a potent inhibitor of *T. cruzi* GAPDH.



Scheme 1. Synthetic route for the preparation of anacardic acid derivatives.

Table 1
Anacardic acid analogues synthesized



7 a-e **8-11**

Compound	*n*	R^1	R^2	R^3
7a–c	4,6,8	OTHP	Et	Me
7d–e	6,9	CH_3	Et	Me
8a–c	4,6,8	OTHP	Et	Me
8d–e	6,9	CH_3	Et	Me
9a–c	4,6,8	OH	Et	H
9d–e	6,9	CH_3	Et	H
10a–c	4,6,8	OH	H	H
10d–e	6,9	CH_3	H	H
11	5	CHO	H	H

Table 2
IC_{50} values for a series of synthetic anacardic acid derivatives as inhibitors of *T. cruzi* GAPDH

Compound	IC_{50}[a] (μM)
1	28 ± 3
7a–e	>300
8a–e	>250
9a–d	>250
9e	215 ± 17
10a	>250
10b	>250
10c	240 ± 22
10d	200 ± 17
10e	55 ± 5
11	>250

[a] The values represent means of at three individual experiments ±SD.

are compounds **1** and **10e** (with IC_{50} values of 28 and 55 μM, respectively), which were then selected for further enzyme kinetic studies. Firstly, these compounds were confirmed to be authentic inhibitors of the target enzyme, since nonspecific inhibition has been discarded as enzyme inhibition was not affected in all cases in the presence of 0.01% Triton X-100 (results not shown). If aggregates formed by a promiscuous compound are responsible for enzyme inhibition, removal of the aggregates from a solution of the compound would decrease inhibition. Therefore, these results eliminate possible mechanisms of inhibition through the interaction of aggregates of many compounds molecules with *T. cruzi* GAPDH, rather than the binding of the individual molecules. This so called promiscuous inhibition generally involves hits from virtual and high-throughput screening as well as some natural products and their synthetic small molecule derivatives.[41]

To explore the mechanism of inhibition in more detail, we have determined K_i values and the type of inhibition with respect to the physiological substrates G-3-P and NAD^+, employing the two compounds (**1** and **10e**) with the lowest IC_{50} values for *T. cruzi* GAPDH (Table 3 and Fig. 2).

The results shown in Table 3 and Figure 2 indicate that the inhibition of *T. cruzi* GAPDH was found to be noncompetitive with respect to both substrates. Noncompetitive inhibitors have binding affinity for both free enzyme and the enzyme–substrate complex. In this situation, dissociation constants must be defined for the binary enzyme-inhibitor complex (K_i) and the ternary *ESI* complex (αK_i). Data for the most potent *T. cruzi* GAPDH inhibitors **1** and **10e** are shown in the Lineweaver–Burk (or double-reciprocal) plots in Figure 2. K_i and αK_i values obtained for these noncompetitive inhibitors are in the low micromolar range (Table 3), and were determined from the collected data employing the Sigma-Plot enzyme kinetics module. Lineweaver–Burk plots showed intercepts of all lines (obtained at different inhibitor concentrations) in the upper left quadrant for the cofactor NAD^+ (similar plots were observed for the substrate G-3-P). Both the slope and the *y* intercept of the double-reciprocal plot were affected by the presence of the noncompetitive inhibitors. The pattern of lines seen when the plots for varying inhibitor concentrations are overlaid depend essentially on the value of α. It can be seen in Figure 2 that the lines intersected at a value of 1/[S] less than zero and a value of $1/v$ greater than zero.

Table 3
Values K_i and αK_i for the noncompetitive inhibitors of *T. cruzi* GAPDH.

Inhibitor	NAD^+		G-3-P	
	K_i (μM)	αK_i (μM)	K_i (μM)	αK_i (μM)
1	4	6	2	4
10e	5	43	4	38

Therefore, when $\alpha > 1$, the inhibitor preferentially binds to the free enzyme, as confirmed by the dissociation constants depicted in Table 3. It is worth noting that noncompetitive inhibitors do not compete with substrate for binding to the free enzyme, they bind to the enzyme at a site distinct from the active site. When the inhibitor displays finite but unequal affinity for the two enzyme forms, they are also sometimes called mixed inhibitors, but this term should be avoided in inhibitors design.[42]

The inhibitors in the present study represent a new chemical diversity for the target enzyme, and have been shown to act noncompetitively in the presence of both physiological substrates. Taken together our data indicate that potency and selectivity can be achieved by inhibitors that bind differentially to the enzyme-substrate complex and free enzyme.

3. Experimental

All commercially available reagents were purchased from Aldrich Chemical Co. Reagents and solvents were purified when necessary according to the usual procedures described in the literature. ^1H and ^{13}C NMR spectra were recorded on a Bruker ARX-200 (200 and 50 MHz, respectively). The IR spectra refer to films and were measured on a Bomem M102 spectrometer. Mass Spectra were recorded on a Shimadzu GCMS-QP5000. Analytical thin-layer chromatography was performed on a 0.25-μm film of silica gel containing fluorescent indicator UV254 supported on an aluminum sheet (Sigma–Aldrich). Flash column chromatography was performed using silica gel (Kieselgel 60, 230–400 mesh, E. Merck). Gas chromatography was performed in a Shimadzu GC-17A with H_2 as carrier and using a DB-5 column. Melting points were performed in Microquímica MQAPF—301. The rotation locular counter-current chromatography was performed using a RLCC-100 apparatus (Tokyo Rikakikai, Tokyo, Japan), which consisted of 16 columns (45 cm × 11 mm ID) divided by centrally perforated PFTE disks into 37 loculi each. Injections were made manually by filling the RLCC loop volume (3 mL). The flow-rate was 90 mL/h, the rotation was 60–70 rpm and the slope 40°. The experiment was carried out at 22 °C.

3.1. Isolation of ancardic acids mixture

The cashew-nut shell (400 g) was extracted with hexane followed by dichloromethane and methanol (1 L, three times for each solvent, for three days). The dichloromethane extract (32.8 g) was analyzed by ^1H NMR and showed to be rich in anacardic acids, cardols and cardanols. This extract was fractionated by RLCC. The system of phases used was hexane/methanol/water (10:9:1). The

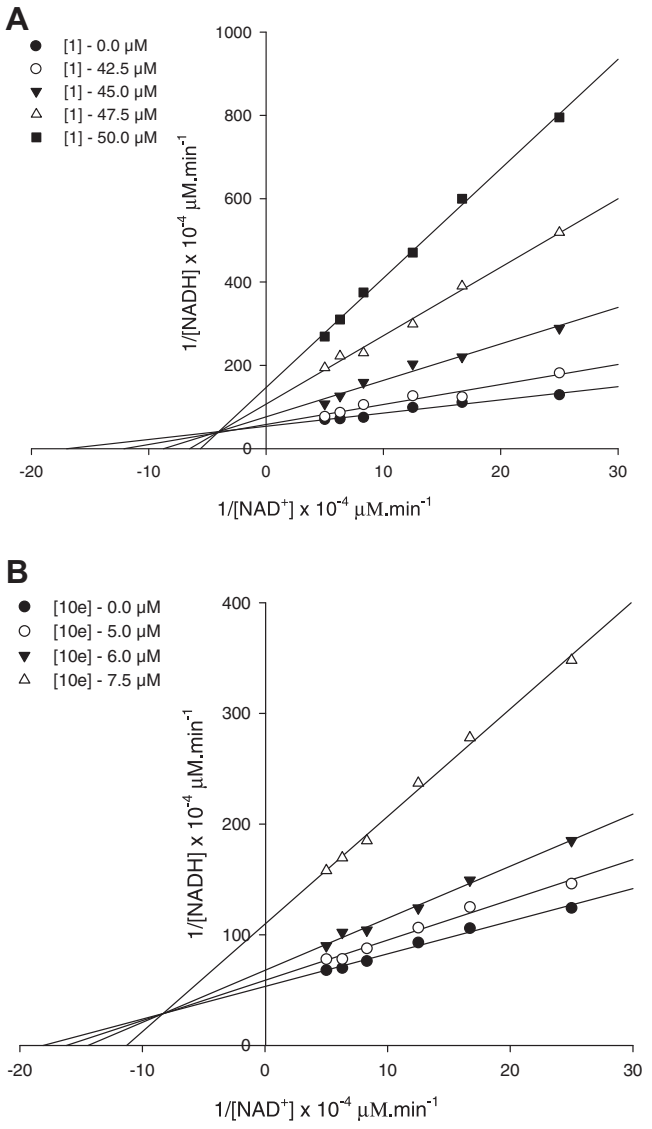


Figure 2. Lineweaver-Burk plots showing that compounds **1** (A) and **10e** (B) inhibits *T. cruzi* GAPDH noncompetitively with respect to NAD^+.

stationary phase was hexane and the mobile phase was methanol/water. A solution of dichloromethane extract (1.0 g) in system phases (3 mL) was injected and eluted in the descending mode with the lipophilic phase. The eluate containing anacardic acids was divided into five fractions. The aqueous extracts were evaporated to dryness and then each fraction was analyzed by ^1H NMR. The fraction containing a mixture of anacardic acids (200 mg) was hydrogenated using H_2 and Pd/C (10%) in methanol during 36 h. After that, the solution was filtrated in Celite and saturated anacardic acid **1** was obtained. IR (ν_{max}/cm^{-1}): 3175, 1630, 1680. ^1H NMR (200 MHz, $CDCl_3$): δ 11.05 (s, 1H); 7.35 (t, 1H , *J* 8.0 Hz); 6.86 (dd, 1H, *J* 0.9, 8.0 Hz); 6.77 (dd, 1H, *J* 0.9, 8.0 Hz); 2.97 (d, 2H, *J* 8.0 Hz); 1.20–1.60 (m, 6H); 0.88 (t, 3 H, *J* 6.0 Hz). ^{13}C NMR (50 MHz, $CDCl_3$): δ 176.0; 163.7; 147.9; 135.4; 122.9; 116.0; 110.8; 36.6; 32.2; 29.7; 22.8; 14.3.

3.2. (3-Methoxyphenyl)-methanol (2)

To a solution of *m*-anisaldeyde (2.03 g, 0.015 mol) in dry methanol (10 mL) was added $NaBH_4$ (0.14 g, 3.75 mmol) at 0 °C. After stirring for 2 h the mixture was quenched with water and extracted with EtOAc. The organic layer was dried with Na_2SO_4 and evaporated to yield an oil. The crude oil was purified by flash chromatography (SiO_2, 6:4 hexane/EtOAc as eluent) to afford **2** (1.89 g, 99%) as a pure yellow oil. IR (ν_{max}/cm^{-1}): 3429, 1596, 1035, 858, 784. ^1H NMR (200 MHz, $CDCl_3$): δ 7.23 (t, 1H, *J* 8.0 Hz); 6.90–6.77 (m, 3H); 4.58 (s, 2H); 3.76 (s, 3H); 2.65 (br s, 1H). MS (*m/z*): 138 (M^+) (base peak), 121, 109, 105, 79, 77 and 51.

3.3. 1-Chloromethyl-3-methoxybenzene (3)

To a mixture of **2** (1.50 g, 0.011 mol) and pyridine (0.44 mL, 5.5 mmol) in benzene (9 mL) was added thionyl chloride (5.62 mL, 0.077 mol) dropwise at 0 °C. After stirring for 2 h the mixture was quenched with water and saturated sodium bicarbonate solution and extracted with EtOAc. The combined organic phase was washed with brine, dried (Na_2SO_4) and evaporated to yield an oil. The crude oil was purified by flash chromatography (SiO_2, 8:2 hexane/EtOAc as eluent) to afford **3** (1.16 g, 96%) as a pure yellow oil. IR (ν_{max}/cm^{-1}): 2960, 2837, 1602, 1490, 1274, 711. ^1H NMR (200 MHz, $CDCl_3$): δ 7.27 (t, *J* 8.0 Hz, 1H); 6.99–6.92 (m, 2H); 6.85 (ddd, 1H, *J* 1.0, 2.5, 8.0 Hz); 4.56 (s, 2H); 3.81 (s, 3H). MS (*m/z*): 158 (M^{+2}), 156 (M^+), 121 (base peak), 106, 92, 91, 77, 51.

3.4. 3-Methoxy-*N*,*N*-dimethylbenzylamine (4)

To a solution of **3** (2.5 g, 0.016 mol) in CH_2Cl_2 (12.5 mL) was added dimethylamine (12.5 mL, 0.24 mol) at 0 °C. The mixture was stirred for 24 h at room temperature and then quenched with water and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and evaporated to yield an oil. Distillation gave **4** (2.5 g, 94%) as a pure yellow oil, bp 43–49 °C/0.12 Torr. IR (ν_{max}/cm^{-1}): 2943, 2775, 1602, 1488, 1269, 1043, 783. ^1H NMR (200 MHz, $CDCl_3$): δ 7.22 (t, 1H, *J* 8.0 Hz); 6.90-6.87 (m, 2H); 6.80 (ddd, 1H, *J* 1.0, 2.5, 8.0 Hz); 3.80 (s, 3H); 3.39 (s, 2H); 2.24 (s, 6H). MS (*m/z*): 165 (M^+), 136, 122, 107, 92, 91, 65, 77, 58 (base peak).

3.5. Ethyl 6-chloromethyl-2-methoxybenzoate (5)

To a stirred solution of **4** (1.0 g, 6.5 mmol) in dry THF (20 mL) was added *n*-butyllithium (0.91 M hexane solution, 9.3 mL, 8.5 mmol) under nitrogen atmosphere at 0 °C. After the solution have been stirred at room temperature for 1 h, ethyl chloroformate (4.25 mL, 34.5 mmol) was added at −78 °C and the mixture was stirred at the same temperature for 1 h and was allowed to stand at room temperature overnight. The mixture was quenched with water, extracted with CH_2Cl_2 and the extract was concentrated to yield an oil. After purification by distillation of byproducts and flash chromatography (SiO_2, 8:1:1 hexane/EtOAc/CH_2Cl_2 as eluent) the pure product **5** was obtained (0.58 g, 42%) as a pale yellow oil, bp 105–112 °C/0.55 Torr. IR (ν_{max}/cm^{-1}): 2979, 1820, 1735, 1473, 1272, 732. ^1H NMR (200 MHz, $CDCl_3$) δ: 7.36 (t, 1H, *J* 8.0 Hz); 7.03 (d, 1H, *J* 8.0 Hz); 6.92 (d, 1H, *J* 8.0 Hz); 4.61 (s, 2H); 4.44 (q, 2H, *J* 8.0 Hz); 3.85 (s, 3H); 1.40 (t, 3H, *J* 8.0 Hz). MS (*m/z*): 228 (M^+), 193, 185, 184, 183, 182 (base peak), 147, 118, 105, 77, 51.

3.6. 2-Ethoxycarbonyl-3-methoxybenzyltriphenylphosphonium chloride (6)

A solution of **5** (1.43 g, 6.3 mmol) and triphenylphosphine (2.14 g, 6.3 mmol) in dry acetonitrile (10 mL) was kept under reflux for 72 h. The solvent was removed and the residue was triturated with dry ether, filtered and evaporated under vacuum to afford **6** (2.52 g, 82%) as a white powder, mp 209–213 °C. IR (nujol, ν_{max}/cm^{-1}): 542, 798, 1284, 1439, 1712. ^1H NMR (200 MHz, $CDCl_3$)

δ: 7.83–7.71 (m, 3H); 7.65–7.55 (m, 12H); 7.26 (t, 1H, *J* 8.0 Hz); 7.00 (dd, 1H, *J* 2.0, 8.0 Hz); 6,91 (dd, 1H, *J* 2.0, 8.0 Hz); 5.41 (d, 2H, *J* 14.0 Hz); 4.06 (q, 2H, *J* 8.0 Hz); 3.77 (s, 3H); 1,20 (t, 3 H, *J* 8.0 Hz).

3.6.1. Compounds 7a–e

To a suspension of the phosphonium salt **6** (500 mg, 1.02 mmol) in dry THF (3.06 mL) was added dropwise lithium bis(trimethylsilyl)amide (1 M hexane solution, 1.53 mL, 1.53 mmol) at −78 °C under nitrogen atmosphere. After the solution has been stirred at −78 °C for 30 min, a solution of the aldehyde (1.22 mmol) in dry THF (3.06 mL) was added dropwise and then allowed to warm to room temperature. After removal of the solvent, the residue was diluted with hexane and the resulting precipitates were filtered. The filtrate was concentrated and after purification by distillation and chromatography on silica gel (95:5 then 1:1 hexane/EtOAc as eluent) the pure products **7a–e** were obtained as a pale yellow oil.

Compound ***7a***. (65 mg; 18% yield). IR (ν_{max}/cm^{-1}): 2939, 2867, 1731, 1575, 1471, 1365, 1267, 1076, 1022, 869, 736. ^{1}H NMR (200 MHz, $CDCl_3$) δ: *E* isomer: 7.27 (t, 1H, *J* 8.0 Hz); 7.10 (d, 1H, *J* 8.0 Hz); 6.86 (d, 1H, *J* 8.0 Hz); 6.41–6.31 (m, 1H); 6.20 (dt, 1 H, *J* 6.2, 15.6 Hz); 4.59–4.53 (m, 1H); 4.41 (q, 2 H, *J* 7.1 Hz); 3.82 (s, 3H); 3.78–3.62 (m, 2 H); 3.55–3.32 (m, 2 H); 2.24–2.17 (m, 2H); 1.75–1.47 (m, 12H); 1.38 (t, 3H, *J* 7.1 Hz). *Z* isomer: 7.29 (t, 1H, *J* 8.0 Hz); 6.80–6.75 (m, 2H); 6.41–6.31 (m, 1H); 5.72 (dt, 1H, *J* 7.4, 11.5 Hz); 4.59-4.53 (m, 1H); 4.36 (q, 2 H, *J* 7.1 Hz); 3.83 (s, 3H); 3.78–3.62 (m, 2H); 3.55–3.32 (m, 2H); 2.24–2.17 (m, 2H); 1.75–1.47 (m, 12H); 1.34 (t, 3H, *J* 7.1 Hz). ^{13}C NMR (50 MHz, $CDCl_3$) δ: *E* isomer: 168.1; 156.3; 136.4; 134.1; 130.0; 126.4; 121.5; 117.7; 109.3; 98.8; 67.3; 62.2; 61.1; 55.9; 32.9; 30.7; 29.2; 25.8; 25.5; 19.6; 14.2. MS (*m/z*): 278, 232, 187, 115, 85 (base peak), 57.

Compound ***7b***. (143 mg; 36% yield). IR (ν_{max}/cm^{-1}): 2935, 2856, 1731, 1585, 1577, 1471, 1267, 1110, 1068, 1033, 811. ^{1}H NMR (200 MHz, $CDCl_3$) δ: *E* isomer: 7.27 (t, 1H, *J* 8.0 Hz); 7.09 (d, 1H, *J* 8.0 Hz); 6.86 (d, 1H, *J* 8.0 Hz); 6.40–6.30 (m, 1H); 6.19 (dt, 1H, *J* 6.0, 15.6 Hz); 4.59–4.56 (m, 1H), 4.40 (q, 2 H, *J* 7.1 Hz); 3.82 (s, 3H); 3.77–3.65 (m, 2H); 3.54–3.33 (m, 2H); 2.20–2.13 (m, 2H); 1.75–1.46 (m, 11H); 1.38 (t, 3H, *J* 7.1 Hz), 1.42–1.30 (m, 3H). *Z* isomer: 7.29 (t, 1H, *J* 8.0 Hz); 6.79–6.75 (m, 2H); 6.40–6.30 (m, 1H); 5.71 (dt, 1H, *J* 7.4, 11.5 Hz); 4.59–4.56 (m, 1H); 4.35 (q, 2 H, *J* 7.1 Hz); 3.83 (s, 3H); 3.77–3.65 (m, 2H); 3.54–3.33 (m, 2H); 2.20–2.13 (m, 2H); 1.75–1.46 (m, 11H); 1.34 (t, 3H, *J* 7.1 Hz); 1.42–1.30 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: *E* isomer: 168.1; 156.4; 136.5; 134.4; 130.0; 126.1; 121.5; 117.7; 109.2; 98.8; 67.6; 62.3; 61.1; 55.9; 33.1; 30.8; 29.7; 29.6; 29.1; 26.1; 25.5; 19.7; 14.3. MS (*m/z*): 260, 187, 174, 115, 85, 55 (base peak).

Compound ***7c***. (105 mg; 24% yield). IR (ν_{max}/cm^{-1}): 2929, 2854, 1731, 1575, 1471, 1268, 1110, 1068, 1031, 810, 734. ^{1}H NMR (200 MHz, $CDCl_3$) δ: *E* isomer: 7.26 (t, 1H, *J* 8.0 Hz); 7.09 (d, 1H, *J* 8.0 Hz); 6.85 (d, 1H, *J* 8.0 Hz); 6.39–6.30 (m, 1H); 6.19 (dt, 1H, *J* 6.2, 15.6 Hz); 4.60–4.53 (m, 1H), 4.40 (q, 2 H, *J* 7.1 Hz); 3.81 (s, 3H); 3.75–3.66 (m, 2H); 3.54–3.31 (m, 2H); 2.16 (qui, 2H, *J* 6.2 Hz); 1.78–1.47 (m, 8H); 1.38 (t, 3H, *J* 7.1 Hz); 1.41–1.22 (m, 10H). *Z* isomer: 7.29 (t, 1H, *J* 8.0 Hz); 6.79–6.74 (m, 2H); 6.39–6.30 (m, 1H); 5.71 (dt, 1 H, *J* 7.4, 11.5 Hz); 4.60–4.53 (m, 1H); 4.35 (q, 2 H, *J* 7.1 Hz); 3.82 (s, 3H); 3.75–3.66 (m, 2H); 3.54–3.31 (m, 2H); 2.16 (qui, 2H, *J* 7.4 Hz); 1.78–1.47 (m, 8H); 1.34 (t, 3H, *J* 7.1 Hz), 1.41–1.22 (m, 10H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: *E* isomer: 168.1; 156.3; 136.5; 135.1; 130.0; 126.1; 121.5; 117.7; 109.2; 98.8; 67.6; 62.3; 61.1; 55.9; 33.1; 30.8; 29.7; 29.4 (2C); 29.1; 28.5; 26.2; 25.5; 19.6; 14.2. MS (*m/z*): 334, 288, 187, 174 (base peak), 115, 91, 55.

Compound ***7d***. (160 mg; 51% yield). IR (ν_{max}/cm^{-1}): 2952, 2925, 2854, 1733, 1583, 1469, 1365, 1267, 1107, 1072, 721 and 541 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$) δ: *E* isomer: 7.25 (t, 1H, *J* 8.0 Hz); 7.09 (d, 1H, *J* 8.0 Hz); 6.86 (d, 1 H, *J* 8.0 Hz); 6.40–6.30 (m, 1H); 6.20 (dt, 1H, *J* 6.2, 15.6 Hz); 4.40 (q, 2 H, *J* 7.1 Hz); 3.80 (s, 3H); 2.16 (qui, 2H, *J* 6.2 Hz); 1.37 (t, 3H, *J* 7.1 Hz); 1.50–1.15 (m, 10H); 0.93–0.83 (m, 3H). *Z* isomer: 7.24 (t, 1H, *J* 8.0 Hz); 6.82–6.73 (m, 2H); 6.40–6.30 (m, 1H); 5.71 (dt, 1H, *J* 7.4, 11.5 Hz); 4.35 (q, 2 H, *J* 7.1 Hz); 3.80 (s, 3H); 2.16 (qui, 2H, *J* 7.4 Hz); 1.34 (t, 3H, *J* 7.1 Hz); 1.50–1.15 (m, 10H); 0.93–0.83 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: *E* isomer: 168.1; 156.3; 136.5; 134.5; 130.0; 126.0; 121.5; 117.7; 109.2; 61.1; 55.9; 33.1; 31.8; 29.6; 29.1; 28.5; 22.6; 14.2; 14.0. MS (*m/z*): 304 (M$^+$), 174 (base peak), 132, 115, 55, 43, 41.

Compound ***7e***. (110 mg; 31% yield). IR (ν_{max}/cm^{-1}): 2954, 2923, 2854, 1731, 1577, 1469, 1267, 1108, 1066, 730. ^{1}H NMR (200 MHz, $CDCl_3$) δ: *E* isomer: 7.26 (t, 1H, *J* 8.0 Hz); 7.10 (d, 1H, *J* 8.0 Hz); 6.86 (d, 1H. *J* 8,0 Hz); 6.39–6.30 (m, 1H); 6.19 (dt, 1H, *J* 6.2, 15.6 Hz); 4.40 (q, 2 H, *J* 7.1 Hz); 3.82 (s, 3H); 2.16 (qui, 2H, *J* 6.2 Hz); 1.38 (t, 3H, *J* 7.1 Hz); 1.39–1.24 (m, 18H); 0.91–0.85 (m, 3H). *Z* isomer: 7.29 (t, 1H, *J* 8.0 Hz); 6.83–6.75 (m, 2H); 6.39–6.30 (m, 1H); 5.71 (dt, 1H, *J* 7.4, 11.5 Hz); 4.35 (q, 2 H, *J* 7.1 Hz); 3.83 (s, 3H); 2.16 (qui, 2H, *J* 7.4 Hz); 2.30–2.10 (m, 2H); 1.34 (t, 3H, *J* 7.1 Hz); 1.39–1.24 (m, 18H); 0.91–0.85 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: *E* isomer: 168.1; 156.3; 136.5; 134.5; 130.0; 126.0; 121.5; 117.7; 109.2; 61.1; 55.9; 33.1; 31.9; 29.64; 29.55; 29.3; 29.2; 29.1; 28.5; 22.6; 14.2; 14.0. MS (*m/z*): 346 (M$^+$), 205, 174 (base peak), 147, 115, 55.

3.6.2. Compounds 8a–e

A mixture of the olefin **7a–e** (0.25 mmol) and 5% Pd/C (5.2 mg; 0.025 mmol) in hexane (5.25 mL) was stirred for 2 h under hydrogen atmosphere. The catalyst was filtered through a short column of Celite and the filtered was concentrated to afford pure **8a-e** as a pale yellow oil.

Compound ***8a.*** (90.3 mg; 98% yield). IR (ν_{max}/cm^{-1}): 2937, 2864, 1731, 1583, 1471, 1365, 1267, 1118, 1074, 1024, 869. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.25 (t, 1H, *J* 8.0 Hz); 6.80 (d, 1H, *J* 8.0 Hz); 6.75 (d, 1H, *J* 8.0 Hz); 4.60–4.52 (m, 1H); 4.38 (q, 2H, *J* 7.1 Hz); 3.81 (s, 3H); 3.74–3.66 (m, 1H); 3.51–3.34 (m, 3H); 2.56 (t, 2H, *J* 7.6 Hz); 1.69–1.53 (m, 10H); 1.37 (t, 3H, *J* 7.1 Hz); 1.40–1.33 (m, 4H). ^{13}C NMR (50 MHz, $CDCl_3$) δ:168.3; 156.2; 141.1; 130.0; 123.8; 121.4; 108.4; 98.8; 67.6; 62.2; 61.0; 55.8; 33.3; 31.1; 30.7; 29.6; 29.3; 26.0; 25.4; 19.6; 14.2. MS (*m/z*): 280, 234, 161, 121, 85 (base peak), 55.

Compound ***8b***. (94 mg; 97% yield). IR (ν_{max}/cm^{-1}): 2935, 2858, 2360, 2337, 1731, 1585, 1469, 1268, 1118, 1076, 1033. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.26 (t, 1H, *J* 8.0 Hz); 6.81 (d, 1H, *J* 8.0); 6.75 (d, 1H, *J* 8.0 Hz); 4.59–4.55 (m, 1H); 4.39 (q, 2H, *J* 7.1 Hz); 3.81 (s, 3H); 3.78–3.66 (m, 2H); 3.55–3.31 (m, 2H); 2.55 (t, 2H, *J* 7.6 Hz); 1.37 (t, 3H, *J* 7.1 Hz); 1.49–1.23 (m, 18H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.3; 156.2; 141.1; 130.0; 123.8; 121.4; 108.4; 98.8; 67.6; 62.3; 61.0; 55.8; 33.4; 31.2; 30.8; 29.7; 29.5; 29.3(2C); 26.2; 25.5; 19.6; 14.2. MS (*m/z*): 392, 262,194,175,161 (base peak), 121, 85, 55.

Compound ***8c***. (103 mg; 98% yield). IR (ν_{max}/cm^{-1}): 2927, 2854, 1731, 1583, 1469, 1267, 1117, 1074, 1033, 763. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.25 (t, 1H, *J* 8.0 Hz); 6.80 (d, 1H, *J* 8.0 Hz); 6.75 (d, 1H, *J* 8.0 Hz); 4.59–4.55 (m, 1H); 4.39 (q, 2H, *J* 7.1 Hz); 3.80 (s, 3H); 3.75–3.58 (m, 2H); 3.54–3.35 (m, 2H); 2.55 (t, 2H, *J* 7.6 Hz); 1.65–1.47 (m, 9H); 1.37 (t, 3H, *J* 7.1 Hz); 1.42–1.24 (m, 13H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.3; 156.2; 141.1; 130.0; 123.8; 121.4; 108.4; 98.8; 67.6; 62.2; 60.9; 55.8; 33.4; 31.1; 30.7; 29.7; 29.5 (2C); 29.39 (2C); 29.36; 26.2; 25.5; 19.6; 14.2. MS (*m/z*): 334, 288, 187, 174 (base peak), 115, 67, 55.

Compound ***8d***. (72 mg; 94% yield). IR (ν_{max}/cm^{-1}): 2954, 2925, 2854, 1731, 1583, 1469, 1267, 1107, 1074, 748. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.23 (t, 1H, *J* 8.0 Hz); 6.80 (d, 1H, *J* 8.0 Hz); 6.73 (d, 1H, *J* 8.0 Hz); 4.38 (q, 2H, *J* 7.1 Hz); 3.78 (s, 3H); 2.55 (t, 2H, *J* 7.6 Hz); 1.64–1.55 (m, 2H); 1.36 (t, 3H, *J* 7.1 Hz); 1.40–1.20 (m,

12H); 0.92–0.84 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$); δ: 168.2; 156.1; 141.0; 129.9; 123.8; 121.3; 108.3; 60.8; 55.7; 33.3; 31.7; 31.1; 29.41; 29.36; 29.3; 29.1; 22.5; 14.1; 13.9. MS (*m/z*): 306 (M^+), 261, 194, 161 (base peak), 121, 91, 77, 55.

Compound ***8e***. (83.1 mg; 95% yield). IR (ν_{max}/cm^{-1}): 2923, 2852, 1731, 1585, 1469, 1267, 1107, 1074, 746. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.25 (t, 1H, *J* 8.0 Hz); 6.81 (d, 1H, *J* 8.0 Hz); 6.75 (d, 1H, *J* 8.0 Hz); 4.39 (q, 2H, *J* 7.1 Hz); 3.81 (s, 3H); 2.55 (t, 2H, *J* 7.6 Hz); 1.65–1.55 (m, 2H); 1.37 (t, 3H, *J* 7.1 Hz); 1.40–1.22 (m, 20H); 0.91–0.84 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.4; 156.2; 141.2; 130.0; 123.9; 121.5; 108.4; 61.0; 55.8; 33.4; 31.9; 31.2; 29.62; 29.54; 29.5 (2C); 29.4; 29.3; 22.6; 14.2; 14.0. MS (*m/z*): 348 (M^+); 303; 175; 161 (base peak); 147; 121; 55.

3.6.3. Compounds 9a–c

To a solution of **8a–c** (0.25 mmol) in dry CH_2Cl_2 (10 mL) was added $AlCl_3$ (1.30 g; 9.7 mmol) under nitrogen atmosphere. After stirring for 14 h at room temperature the mixture was quenched with water and extracted with EtOAc. The organic layer was dried (Na_2SO_4) and evaporated to give **9a–c** as a pale yellow oil.

3.6.4. Compounds 9d–e

To a solution of **8d–e** (0.12 mmol) in dry CH_2Cl_2 (0.03 mL) was added BBr_3 (1 M solution in CH_2Cl_2, 5.2 μL) dropwise at −40 °C under nitrogen atmosphere. After stirring for 2 h at room temperature the mixture was poured into ice water and extracted with CH_2Cl_2. The organic layer was dried (Na_2SO_4) and evaporated to give **9d-e** as a pale yellow oil.

Compound ***9a***. (26 mg; 42% yield). IR (ν_{max}/cm^{-1}): 3406, 2933, 2858, 1728, 1654, 1606, 1450, 1373, 1249, 1213, 1058, 1027, 709. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 11.22 (s, 1H); 7.28 (t, 1H, *J* 7.6 Hz); 6.83 (dd, 1H, *J* 1.2, 8.3 Hz); 6.71 (dd, 1H, *J* 1.2, 7.5 Hz); 4.43 (q, 2H, *J* 7.1 Hz); 3.64 (t, 2 H, *J* 6.4 Hz); 2.95–2.87 (m, 2H); 1.60–1.54 (m, 5H); 1.43 (t, 3H, *J* 7.1 Hz); 1.48–1.35 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 171.5; 162.6; 145.9; 134.0; 122.4; 115.6; 112.0; 62.9; 61.6; 36.5; 32.7; 32.1; 29.6; 25.7; 14.1. MS (*m/z*): 266 (M^+), 220, 147, 134 (base peak), 105, 91, 77, 55.

Compound ***9b***. (47 mg; 64% yield). IR (ν_{max}/cm^{-1}): 3444, 2927, 2854, 1658, 1608, 1450, 1249, 1064, 813 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 11.22 (s, 1H); 7.28 (dd, 1H, *J* 7.6, 8.3 Hz); 6.83 (dd, 1H, *J* 1.2, 8.3 Hz); 6.71 (dd, 1H, *J* 1.2, 7.6 Hz); 4.44 (q, 2H, *J* 7.1 Hz); 3.64 (t, 2 H, *J* 6.6 Hz); 2.92–2.88 (m, 2H); 1.58–1.51 (m, 4H); 1.43 (t, 3H, *J* 7.1 Hz); 1.37–1.33 (m, 8H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 171.5; 162.6; 146.1; 134.0; 122.4; 115.6; 112.0; 63.0; 61.6; 36.7; 32.7; 32.2; 29.8; 29.5; 29.4; 25.7; 14.1. MS (*m/z*): 294 (M^+), 248, 197, 152, 147 (base peak), 134, 105, 77, 55.

Compound ***9c***. (32 mg; 42% yield). IR (ν_{max}/cm^{-1}): 3402, 2923, 2852, 1726, 1654, 1606, 1450, 1373, 1249, 1213, 1103, 1058, 1022, 817, 709 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 11.23 (s, 1H); 7.28 (t, 1H, *J* 7.6 Hz); 6.83 (dd, 1H, *J* 1.2, 8.3 Hz); 6.71 (dd, 1H, *J* 1.2, 7.6 Hz); 4.44 (q, 2H, *J* 7.1 Hz); 3.64 (t, 2 H, *J* 6.6 Hz); 2.92–2.88 (m, 2H); 1.57–1.53 (m, 4H); 1.43 (t, 3H, *J* 7.1 Hz); 1.36–1.29 (m, 12H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 171.6; 162.6; 146.1; 134.0; 122.5; 115.6; 112.0; 63.0; 61.6; 36.7; 32.8; 32.2; 29.9; 29.6 (2C); 29.5; 29.4; 25.7; 14.1. MS (*m/z*): 322 (M^+), 276, 161, 147 (base peak), 134, 105, 55.

Compound ***9d***. (28.3 mg; 82% yield). IR (ν_{max}/cm^{-1}): 2954, 2925, 2854, 1660, 1606, 1448, 1373, 1311, 1249, 1211, 1118, 709 cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 11.23 (s, 1H); 7.28 (t, 1H, *J* 7.9 Hz); 6.83 (dd, 1H, *J* 1.2, 8.3 Hz); 6.71 (dd, 1H, *J* 1.2, 7.5 Hz); 4.43 (q, 2H, *J* 7.1 Hz); 2.94–2.86 (m, 2 H); 1.43 (t, 3H, *J* 7.1 Hz); 1.48–1.23 (m, 14H); 0.91–0.85 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 171.5; 162.6; 146.2; 134.0; 122.4; 115.6; 112.0; 61.6; 36.7; 32.3; 31.9; 29.9; 29.6; 29.5; 29.3 (2C); 22.6; 14.0. MS (*m/z*): 292 (M^+), 246, 175, 161, 147, 134 (base peak), 133, 107, 105, 91, 77, 55.

Compound ***9e***. (33.7 mg; 83% yield). IR (ν_{max}/cm^{-1}): 2923, 2853, 1663, 1607, 1576, 1448, 1372, 1312, 1249, 1212, 1119, 1020, 816. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.28 (t, 1H, *J* 7.9 Hz); 6.83 (dd, 1H, *J* 1.2, 8.3 Hz); 6.71 (dd, 1H, *J* 1.2, 7.5 Hz); 4.43 (q, 2H, *J* 7.1 Hz); 2.92–2.88 (m, 2 H); 1.58–1.51 (m, 2H); 1.43 (t, 3H, *J* 7.1 Hz); 1.33–1.22 (m, 18H); 0.88 (t, 3H, *J* 6.8 Hz). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 171.6; 162.7; 146.1; 134.0; 122.4; 115.6; 112.0; 61.6; 36.7; 32.3; 31.9; 29.9; 29.7 (3C); 29.6 (3C); 29.3; 22.7; 14.1. MS (*m/z*): 334 (M^+), 288, 262, 288, 180, 161, 147 (base peak), 134, 105, 91, 77, 55.

3.6.5. Compounds 10a–e

A solution of **9a–e** (0.10 mmol) and 20% NaOH solution (0.20 mL) in DMSO (0.20 mL) was kept under reflux at 120 °C for 5 h. The mixture was acidified with 10% HCl and extracted with EtOAc. The organic layer was washed with water, dried (Na_2SO_4) and evaporated to give a pale yellow oil. The crude oil was chromatographed on silica gel (9:1:0.2 hexane/EtOAc/acetic acid as eluent) to afford the anacardic acids and analogues as white powders.

Compound ***10a***. (16 mg; 68% yield). mp 78–79 °C; IR (ν_{max}/cm^{-1}): 3421, 2933, 2858, 1718, 1654, 1602, 1452, 1315, 1247, 1216, 1020, 709. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.22 (t, 1H, *J* 7.8 Hz); 6.73–6.70 (m, 2H); 5.48 (s, 1H); 3.53 (t, 2H, *J* 6.6 Hz); 2.92–2.88 (m, 2H); 1.62–1.49 (m, 5H); 1.39–1.36 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 174.2; 162.2; 146.7; 133.9; 122.8; 115.7; 115.6; 63.0; 36.6; 33.1; 33.2; 30.6; 26.7. MS (*m/z*): 220, 194, 147, 120, 108 (base peak), 91, 77, 55.

Compound ***10b***. (18.1 mg; 72% yield). mp 79–81 °C; IR (ν_{max}/cm^{-1}): 3467, 2923, 2850, 2534, 1708, 1662, 1604, 1454, 1261, 1214, 1091, 802, 732. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.09 (dd, 1H, *J* 7.6, 8.3 Hz); 6.57 (ddd, 2H, *J* 1.2, 7.6, 8.3 Hz); 3.39 (t, 2H, *J* 6.6 Hz); 2.75–2.71 (m, 2H); 1.46–1.34 (m, 4H); 1.27–1.13 (m, 8H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 174.3; 162.2; 146.7; 134.0; 122.9; 115.7; 115.6; 62.9; 36.7; 33.6; 33.2; 30.8; 30.5 (2C); 26.9. MS (*m/z*): 222, 147, 120, 108 (base peak), 77, 55.

Compound ***10c***. (22 mg; 77% yield). mp 80–82 °C; IR (ν_{max}/cm^{-1}): 3474, 3420, 2919, 2849, 1652, 1609, 1448, 1325, 1247, 1215, 736. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.23 (t, 1H, *J* 7.8 Hz); 7.74–6.69 (m, 2H); 3.52 (t, 2H, *J* 6.6 Hz); 2.88–2.85 (m, 2H); 1.59–1.48 (m, 4H); 1.39–1.25 (m, 12H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 174.3; 162.2; 146.7; 134.1; 122.9; 115.7; 115.6; 63.0; 36.7; 33.6; 33.2; 30.8; 30.7; 30.6 (2C); 30.5; 26.9. MS (*m/z*): 276, 250, 161, 147, 120, 108 (base peak), 77, 55.

Compound ***10d***. (20 mg; 85% yield). mp 78–79 °C; IR (ν_{max}/cm^{-1}): 3429, 3056, 2923, 2854, 2596, 1652, 1606, 1450, 1301, 1245, 1211, 707. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 11.09 (br s, 1H); 7.34 (t, 1H, *J* 7.9 Hz); 6.85 (d, 1H, *J* 8.3 Hz); 6.76 (d, 1H, *J* 7.5 Hz); 3.01–2.93 (m, 2H); 1.65–1.53 (m, 2H); 1.40–1.10 (m, 14H); 0.93–0.80 (m, 3H). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 175.3; 163.5; 147.6; 135.0; 122.6; 115.7; 110.7; 36.4; 32.0; 31.9; 29.8; 29.6; 29.5; 29.3; 22.6; 14.0. MS (*m/z*): 220, 121, 108 (base peak), 77, 55.

Compound ***10e***. (24 mg; 85% yield). mp 90–92 °C; IR (ν_{max}/cm^{-1}): 3453. 3415, 3084, 3055, 2916, 2848, 1652, 1603, 1445, 1307, 1247, 1215, 896, 732. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.23 (t, 1H, *J* 7.8 Hz); 6.73–6.69 (m, 2H); 2.89–2.85 (m, 2H); 1.59–1.52 (m, 2H); 1.38–1.24 (m, 18H); 0.88 (t, 3H, *J* 6.9 Hz). ^{13}C NMR (50 MHz, $CDCl_3$) δ: 174.3; 162.2; 146.7; 134.0; 122.9; 115.7; 115.6; 36.7; 33.2; 33.0; 30.9; 30.8; 30.7 (3C); 30.5; 30.4; 23.7; 14.4. MS (*m/z*): 262, 149, 121, 108 (base peak), 77, 55.

3.7. 2-Hydroxy-6-(8-oxooctyl) benzoic acid (11)[41]

To a solution of oxalyl chloride (17.1 mL, 0.19 mmol) in dry CH_2Cl_2 (0.38 mL) at −78 °C was added DMSO (25.0 mL, 0.35 mmol) in dry CH_2Cl_2 (0.38 mL) under nitrogen atmosphere. After stirring for 5 min a solution of alcohol **10b** (35.0 mg, 0.13 mmol) in dry CH_2Cl_2/DMSO (0.38/0.025 mL) was added dropwise. After stirring

for more 5 min, triethylamine was added (0.13 mL, 0.97 mmol) and the mixture was allowed to warm to room temperature. Finally, the solvent was removed, the residue was diluted with ethyl acetate and the resulting precipitates were filtered. The filtrate was concentrated and after a quick purification by chromatography on silica gel (8:2:0.25 hexane/EtOAc/acetic acid as eluent) the pure product was obtained as pale yellow oil (0.025 mg, 73%). mp 78–79 °C; IR (ν_{max}/cm^{-1}): 3421, 2928, 2854, 1716, 1651, 1605, 1575, 1450, 1243, 1221, 1043, 741 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$) δ: 9.76 (t, 1H, *J* 1.7 Hz); 7.34 (t, 1 H, *J* 8.0 Hz); 6.86 (dd, 1H, *J* 1.0, 8.0 Hz); 6.75 (dd, 1H, *J* 1.0, 7.6 Hz); 3.00–2.92 (m, 2H); 2.44 (dt, 2H, *J* 1.7, 7.1 Hz); 1.70–1.51 (m, 4H); 1.45–1.26 (m, 6H). MS (*m*/*z*): 220, 207, 147, 121, 108 (base peak), 77, 55.

3.8. Expression and purification of glycosomal *T. cruzi* GAPDH

Protein expression and purification were carried out as described previously.[10,43] Briefly, culture of *E. coli* with recombinant *T. cruzi* GAPDH was incubated and the expression of this enzyme was then induced under specific conditions. After this procedure the cells were lysed and submitted to a centrifugation. The resulted supernatant was fractionated and the mixture was centrifuged. The supernatant was purified by hydrophobic chromatography and the eluted fractions were subsequently purified by cation-exchange chromatography. The concentration of GAPDH that was further dialyzed against TEA buffer was performed using an Amicon concentrator until achieving the final concentration of 9 mg/mL. The purity of the protein was checked and monitored by SDS–PAGE.

3.9. Kinetic measurements

All enzymatic assays were carried out in triplicate at 25 °C. The reaction mixture contained 100 mM triethanolamine, HCl buffer (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol, 30 mM $NaHAsO_4{\cdot}7H_2O$, 400 μM NAD^+, 20 nM GAPDH, and 300 μM D-glyceraldehyde-3-phosphate. The final reaction volume was 1000 μL, kept under stirring. The biochemical reduction of NAD^+ to NADH was monitored at 340 nm for 8 min using a Cary 100 Bio UV/V is equipped with a Peltier-thermostatted multicell changer and a temperature controller. Values of IC_{50} were independently determined by making rate measurements for at least five inhibitor concentrations. The values represent means of at least three individual experiments. Values of K_i and the type of inhibition were determined for 3 or 4 different inhibitor concentrations. Kinetic parameters and inhibitor modality were estimated from the collected data employing the Sigma-Plot enzyme kinetics module.

3.10. Nonspecific inhibition assay

The possible promiscuous inhibitory mechanism was evaluated by adding Triton X-100 to the reaction mixture during the assays in order to reverse or prevent possible compound association during the experiments. The stock solution 1% (v/v) was freshly prepared in triethanolamine, HCl buffer (pH 7) with EDTA and 2-mercaptoethanol to achieve the equivalent concentration in the cuvette as the same for assay reaction. The volume of this solution (5 μL) was then added to perform 0.01% of Triton in the reaction mixture, a concentration that was preliminary evaluated and showed no interference with enzymatic activity. During the assays this solution was added in the mixture through two procedures. First, the addition occurred right after putting the compound. Second, the compound was added, followed by Triton X-100 and then enzyme. In both cases, the degree of inhibition was kept constant independently if there was Triton or if the mixtures were or not incubated (data not shown), which proved a nonpromiscuous inhibition.

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