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RESEARCH ARTICLE

Synthesis and pharmaceutical properties of *N*-acyloxymethyl prodrugs of Allop with potential anti-trypanosomal activity

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Abstract

We report herein the synthesis, and the physicochemical and pharmacokinetic properties of *N*-acyloxymethyl prodrugs of allopurinol (Allop) (**2a**–**f**). Allop is a compound with activity against *Trypanosoma cruzi*, a causative agent of Chagas disease. Its pathology leads to a huge number of infections and deaths per year, because in addition to many sufferers only having limited access to health services only an inefficient chemotherapy is available. Relevant pharmaceutical properties (pK_a, stability, solubility, lipophilicity, *in vitro* permeability, binding protein, xanthine oxidase binding) were also determined. The results obtained showed that derivatives behave as prodrugs of Allop, since they exhibit improved physicochemical and pharmacokinetic properties relative to their precursor. This behavior turns these compounds into active reservoirs of Allop, and reduces its unfavorable characteristics, so **2a**–**f** compounds are excellent candidates for the treatment of Chagas disease. This work is therefore an important contribution leading to the suppression of Chagas disease.

Keywords

Chagas disease, *N*-acyloxymethyl prodrugs, physicochemical and pharmacokinetic properties, *Trypanosoma cruzi*, trypanosomal activity

History

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Introduction

Chagas disease caused by the protozoan parasite *Trypanosoma cruzi* is considered a neglected disease by the World Health Organization and constitutes an important health problem in the poorest regions. An estimated 8 million people are infected worldwide, mostly in Latin America[1].

After one hundred years of discovering this chronic disease, there are no preventive vaccines and the current chemotherapies rely only on benznidazole (Bz) and nifurtimox that were developed more than 40 years ago. These two compounds are not optimal as currently applied because they show substantial toxicity, require long courses of administration and have inconsistent efficacy. In addition, the production of nifurtimox was discontinued in 1997, first in Brazil, and then in Argentina, Chile and Uruguay. Currently, Bz is the only commercially available treatment in most endemic countries[2]. Today the vector control is the most useful method to prevent Chagas disease[1].

Thus, there is an urgent need for new treatment options and growing interest in drug development for the infection. Research on anti-trypanosomal compounds has been to date based on different strategies[2–4]. One possibility is to exploit the differences in the purine metabolism between *T. cruzi* and host cells. Usually, parasites cannot synthesize the purines necessary for many cellular processes. The purine comes from the host cell and to this end, the parasites have purine uptake and salvage pathways, which are not present in the host, to transport, internalize and metabolize the required substrates[5].

Allopurinol (Allop, Scheme 1), employed for the treatment of hyperuricemia, is a hypoxanthine analog used for the *T. cruzi*'s hypoxanthineguanine-phosphoribosyltransferase (HGPRT) as an alternative substrate. This enzyme can incorporate Allop into the parasite's ribonucleic acid as a non-physiological nucleotide, thus blocking the synthesis of new purine nucleotides[6,7].

The activity of Allop in chemotherapy for Chagas disease has been extensively investigated, but the results are somewhat contradictory[8–11]. In a pilot study, Perez-Mazliah et al. showed that the combination of Allop and Bz induced significant modifications of the T and B cell responses, indicative of a reduction of the parasite burden, and thereby sustained the feasibility of administration of two antiparasitic drugs in the chronic phase of Chagas disease[12].

As mentioned above, it is verified that Allop is a drug with anti-trypanosomal activity, but the versatile responses in humans would be due in part to the infective parasite population, which varies among geographical areas[13], to inadequate blood levels [Allop is converted into oxypurinol by xanthine oxidase (XO), which is not a substrate for HGPRT and has no anti-trypanosomal activity][6] and to its unfavorable physicochemical properties.

In an attempt to improve its performance, we previously studied C6-alkyl derivatives and *N*-acyl prodrugs of Allop, which demonstrated to be effective against *T. cruzi*, but unstable in plasma[14].

Based on this background and considering that Allop is an effective anti-trypanosomal agent, we propose obtaining prodrugs as a valuable tool to overcome limitations of the parent drug. An ideal prodrug has optimal physicochemical properties, such as lipophilicity and solubility, is stable in the gastrointestinal tract,

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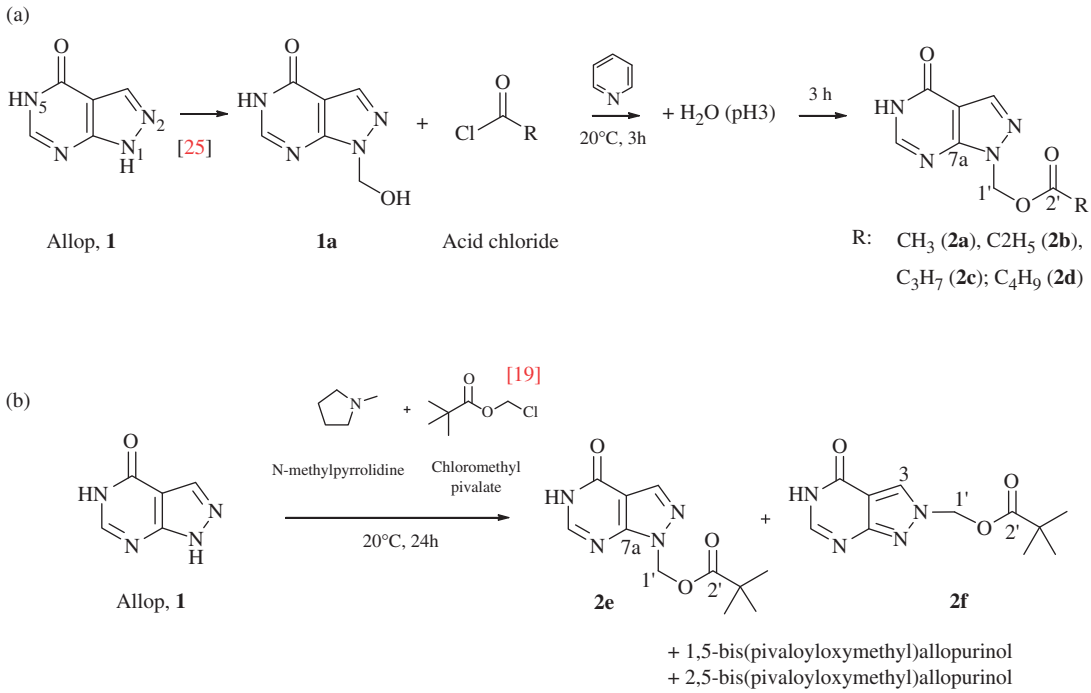

Scheme 1. Synthesis procedures for the development of Allop derivatives (**2a–f**): (a) via 1-(hidroxymethyl)allopurinol (**1a**) and (b) direct synthesis.

releases a promoiety that is nontoxic and regenerates the active drug at an appropriate rate *in vivo*[15].

Therefore, we now suggest the formation of a series of *N*-acyloxymethyl prodrugs of Allop with short, long and branched substituent chains of alkyl (Scheme 1).

For all the foregoing reasons, it was relevant to carry out a thorough study of the physicochemical properties such as pK_a, lipophilicity, solubility, and chemical, enzymatic, and human plasma stability of the new derivatives (some of them were previously reported)[16] as well as of the characteristic properties of their pharmacokinetic behavior (artificial membrane permeability, plasmatic protein binding and affinity for XO). The aim of these studies is to achieve a general understanding of the behavior of prodrugs of Allop and of their characteristics.

Material and methods

Chemicals and reagents

Allop (99% Sigma, St. Louis, MO), formaldehyde (40wp Biopack, Zárate, Argentina), acetyl chloride (98% Sigma), propionyl chloride (98% Aldrich, St. Louis, MO), butyryl chloride (98% Aldrich), valeroyl chloride (98% Aldrich), chloromethyl pivalate (97% Aldrich), *N*-methylpyrrolidine (97% Aldrich). Tris–HCl buffer hydroxymethyl aminomethane (99% Biopack), XO, from bovine milk (XO, 0.11 U mg^{-1} solid, Sigma), Xanthine (≥99% Sigma). Pyridine was purified and dried according to a previously described procedure[17]. Other reagents were of commercial quality (>97%) from freshly opened containers. Acetonitrile, CH_2Cl_2, ethyl acetate, ethanol and other solvents were of analytical grade. The buffer materials of pH 1.2 (HCl (37%) 0.175 mL, NaCl 0.05 g L^{-1}), pH 5.5 for lipophilicity (HCH_2COOH 0.090 mL, HCH_2COONa 1.156 g L^{-1}), pH 5.5 for permeability (KH_2PO_4 1.27 g L^{-1}, Na_2HPO_4 0.13 g L^{-1}), pH 6.8 (KH_2PO_4 6.8 g L^{-1}, NaOH 1.52 g L^{-1}), PBS buffer pH 7.4 (NaCl 8.01 g L^{-1}, KCl 0.20 g L^{-1}, Na_2HPO_4 1.42 g L^{-1}, KH_2PO_4 0.27 g L^{-1}) were of analytical reagent grade.

The SGF and SIF were prepared according to USP specifications[18]. Sodium dodecyl sulfate (SDS) was purchased from Biopack; 1-propanol (analytical grade) was purchased from Cicarelli and methanol (MeOH, HPLC grade) was acquired from Sintorgan. The water used in all studies was of Milli-Q grade (Millipore, Molsheim, France), and solutions and mobile phases were filtered through Millipore filters Type FH (4.5 mm) and degassed under vacuum.

Equipment

High resolution mass spectrometry (HRMS) was performed on a Bruker microTOF-Q II (Billerica, MA), using the electrospray ionization (ESI) positive mode. Nuclear magnetic resonance (NMR) experiments were performed on Bruker Avance II 400, Ultrashield, Frequency ^{1}H NMR 400.16 MHz and ^{13}C NMR 100.62 Hz, Dual BBI Probe, at 25 °C, using DMSO-d_6 (99.8%, Merck) as solvent. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of D_2O (99.9%, Sigma), and chemical shift values are reported in parts per million (δ) relative to tetramethylsilane. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; qua, quartet; quin, quintet; sex, sextuplet. Ultraviolet spectrophotometric (UV) studies were carried out using a Shimadzu Model UV-160A spectrophotometer (Kyoto, Japan), with 1 cm quartz cells. Melting point (mp) and thermal analysis were carried out using differential scanning calorimetry (DSC) 2920 modulated DSC (TA Instrument, New Castle, PE); the temperature axis and the cell constant of the DSC were calibrated with indium, and the thermogravimetric analysis (TG) measurements were performed with a TA Instrument Hi-res TG 2950 (TA Instrument). Weighed samples (1.5–3 mg, C-33 Microbalance, Cahan) were scanned in covered aluminum (Al) pans under a dynamic dry nitrogen atmosphere (50 mL min^{-1}). The pH values were measured with a CRISON GLP-21 pH meter (Modena, Italy) equipped with an Ag/AgCl glass electrode.

Chemistry

*Method A. **2a–d***

For suspension of 1-(hydroxymethyl)allopurinol (**1a** 0.415 g; 2.5 mmol) in anhydrous pyridine (5 mL), acid chloride (5 mmol)

was added. The mixture was stirred at 20 °C for 3 h. Then, acidified water (10 mL) was added and the mixture was stirred for 3 h. The precipitate was collected, and recrystallized.

4-Oxo-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-1-yl-methyl acetate (**2a**). White solid, recrystallization from EtOH (62% yield); $R_f = 0.67$ (CH_2Cl_2/EtOH 8:2 v/v); m.p.: 260 ± 2 °C; HPLC $tr = 3.7$ min, MLC $tr = 2.7$ min. ^{1}H NMR (DMSO-d_6): δ 12.41 (s, 1H, NH-5), 8.20 (s, 1H, H-3), 8.18 (s, 1H, H-6), 6.22 (s, 2H, H-1′), 2.06 (s, 3H, H-3′). ^{13}C NMR (DMSO-d_6): δ 169.6 (CO, C-2′), 157.3 (CO, C-4), 153.6 (C, C-7a), 149.7 (CH, C-6), 136.8 (CH, C-3), 106.9 (C, C-3a), 68.5 (CH_2, C-1′), 20.9 (CH_3, C-3′). HRMS (ESI) calcd for $C_8H_8N_4NaO_3$ $(M + Na)^+$ *m/z*: 231.0487; found: 231.1638. UV (MeOH, pH 5) λ_{max} 250 nm.

4-Oxo-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-1-yl-methyl propionate (**2b**). White solid, recrystallization from EtOH (41% yield); $R_f = 0.70$ (CH_2Cl_2/EtOH 8:2 v/v); m.p.: 239 ± 1 °C; HPLC $tr = 4.7$ min, MLC $tr = 3.7$ min. ^{1}H NMR (DMSO-d_6): δ 12.40 (s, 1H, NH-5), 8.20 (s, 1H, H-3), 8.18 (s, 1H, H-6), 6.23 (s, 2H, H-1′), 2.34 (qua, 2H, H-3′), 1.01 (t, 3H, H-4′). ^{13}C NMR (DMSO-d_6): δ 173.0 (CO, C-2′), 157.6 (CO, C-4), 153.7 (C, C-7a), 149.5 (CH, C-6), 136.7 (CH, C-3), 106.8 (C, C-3a), 68.6 (CH_2, C-1′), 26.9 (CH_2, C-3′), 9.1 (CH_3, C-4′). HRMS (ESI) calcd for $C_9H_{10}N_4NaO_3$ $(M + Na)^+$ *m/z*: 245.0645; found: 245.1904; UV (MeOH, pH 5) λ_{max} 250 nm.

4-Oxo-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-1-yl-methyl butyrate (**2c**). White solid, recrystallization from EtOH (44% yield); $R_f = 0.73$ (CH_2Cl_2/EtOH 8:2 v/v); m.p.: 237 ± 1 °C; HPLC $tr = 6.4$ min, MLC $tr = 5.6$ min. ^{1}H NMR (DMSO-d_6): δ 12.40 (s, 1H, NH-5), 8.20 (s, 1H, H-3), 8.18 (s, 1H, H-6), 6.23 (s, 2H, H-1′), 2.31 (t, 2H, H-3′), 1.52 (sex, 2H, H-4′), 0.85 (t, 3H, H-5′). ^{13}C NMR (DMSO-d_6): δ 172.1 (CO, C-2′), 157.5 (CO, C-4), 153.6 (C, C-7a), 149.6 (CH, C-6), 136.7 (CH, C-3), 106.8 (C, C-3a), 68.4 (CH_2, C-1′), 35.4 (CH_2, C-3′), 18.2 (CH_2, C-4′), 13.7 (CH_3, C-5′). HRMS (ESI) calcd for $C_{10}H_{12}N_4NaO_3$ $(M + Na)^+$ *m/z*: 259.0802; found: 259.2170; UV (MeOH, pH 5) λ_{max} 250 nm.

4-Oxo-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidin-1-yl-methyl pentanoate (**2d**). White solid, recrystallization from EtOH (63% yield); $R_f = 0.75$ (CH_2Cl_2/EtOH 8:2 v/v); m.p.: 232 ± 1 °C; HPLC $tr = 9.8$ min, MLC $tr = 8.5$ min. ^{1}H NMR (DMSO-d_6): δ 12.40 (s, 1H, NH-5), 8.20 (s, 1H, H-3), 8.18 (s, 1H, H-6), 6.23 (s, 2H, H-1′), 2.32 (t, 2H, H-3′), 1.48 (quin, 2H, H-4′), 1.24 (sex, 2H, H-5′), 0.83 (t, 3H, H-6′). ^{13}C NMR (DMSO-d_6): δ 172.3 (CO, C-2′), 157.4 (CO, C-4), 153.7 (C, C-7a), 149.5 (CH, C-6), 136.8 (CH, C-3), 106.8 (C, C-3a), 68.4 (CH_2, C-1′), 33.4 (CH_2, C-3′), 26.7 (CH_2, C-4′), 21.9 (CH_2, C-5′), 14.0 (CH_3, C-6′). HRMS (ESI) calcd for $C_{11}H_{14}N_4NaO_3$ $(M + Na)^+$ *m/z*: 273.0958; found: 273.2437; UV (MeOH, pH 5) λ_{max} 250 nm.

*Method B (**2e** and **f**)*

These derivatives were prepared according to the Taylor and Sloan procedures for 5-fluoruracil[19]. For purification, the crude of reaction was evaporated. The oil that resulted was diluted with CH_2Cl_2, and the solution was washed with aqueous acid and water and then dried over Na_2SO_4. The dried CH_2Cl_2 solution was evaporated and the residue was then purified by column chromatography on silica gel, using initial eluent of CH_2Cl_2 followed by a gradient of solvent until a solution of CH_2Cl_2-ethyl acetate (5:5 v/v). The compounds were collected in the following order: 1,5-bis(pivaloyloxymethyl)allopurinol from CH_2Cl_2; 2,5-bis(pivaloyloxymethyl)allopurinol from CH_2Cl_2–ethyl acetate (9:1 v/v); **1** (pivaloyloxymethyl)allopurinol from CH_2Cl_2–ethyl acetate (7:3 v/v), **2** (pivaloyloxymethyl)allopurinol from CH_2Cl_2-ethyl acetate (5:5 v/v).

4-Oxo-4,5-dihydro-1H-pyrazolo [3,4-d] pyrimidin-1-yl) methyl pivalate (**2e**). White solid (12% yield); R_f = 0.55 (CH_2Cl_2/EtOH 10:1 v/v); m.p.: 196 ± 2 °C; MLC $tr = 7.6$ min. ^{1}H NMR (DMSO-d_6): δ 12.43 (s, 1H, NH-5), 8.19 (s, 1H, H-3), 8.18 (s, 1H, H-6), 6.23 (s, 2H, H-1′), 1.10 (2, 9H, H-4′). ^{13}C NMR (DMSO-d_6): δ 176.7 (CO, C-2′), 157.6 (CO, C-4), 153.7 (C, C-7a), 149.7 (CH, C-6), 136.8 (CH, C-3), 106.9 (C, C-3a), 68.8 (CH_2, C-1′), 38.9 (C, C-3′), 27.1 (CH_3, C-4′). HRMS (ESI) calcd for $C_{11}H_{14}N_4NaO_3$ $(M + Na)^+$ *m/z*: 273.0958; found: 273.2437; UV (MeOH, pH 5) λ_{max} 250 nm.

4-Oxo-4,5-dihydro-2H-pyrazolo[3,4-d]pyrimidin-2-yl) methyl pivalate (**2f**). White solid (24% yield); R_f = 0.40 (CH_2Cl_2/EtOH 10:1 v/v); m.p.: 189 ± 2 °C; MLC $tr = 5.6$ min. ^{1}H NMR (DMSO-d_6): δ 11.84 (s, 1H, NH-5), 8.70 (s, 1H, H-3), 7.99 (s, 1H, H-6), 6.20 (s, 2H, H-1′), 1.10 (2, 9H, H-4′). ^{13}C NMR (DMSO-d_6): δ 177.0 (CO, C-2′), 159.8 (CO, C-4), 159.1 (C, C-7a), 148.3 (CH, C-6), 131.6 (CH, C-3), 108.2 (C, C-3a), 73.9 (CH_2, C-1′), 38.7 (C, C-3′), 27.1 (CH_3, C-4′). HRMS (ESI) calcd for $C_{11}H_{14}N_4NaO_3$ $(M + Na)^+$ *m/z*: 273.0958; found: 273.2437; UV (MeOH, pH 5) λ_{max} 264 nm.

Chromatographic conditions

Analytical thin layer chromatography was performed on percolated plates purchased from Merck (Silica Gel 60 F254). MLC analyses were carried out using an Agilent Technologies Series 1100 apparatus (Palo Alto, CA) equipped with a Phenomenex® column (Torrance, CA), Hypersil ODS 5 µm particle diameter (4.6 × 250 mm); MLC conditions: SDS (0.10 M) – 7.5% (v/v) 1-propanol – NaH_2PO_4/H_3PO_4 (0.01 M, pH 3.0), a flow rate of 1 mL min^{-1}, 25 °C, λ = 250 nm.

Physicochemical properties

Stability studies

Buffers (pH 1.2 and pH 6.8), SGF, SIF and human plasma. The stock solutions (1.0×10^{-4} mol mL^{-1}) of each compound were prepared in DMSO prior to use. Then, 100 µL of the stock solution was added to a vial containing 1900 µL of buffer or matrix (gastric or intestinal fluid) to obtain the work solutions. Afterward, the vials containing the samples were placed in a water bath at 37 °C throughout the experiment. The experiments in human plasma were performed under the experimental conditions suggested by Di et al.[20] Human plasma (unit No. 65261) was generously supplied by Instituto de Hematología, Hemoterapia y Banco de Sangre, Universidad Nacional de Córdoba, Argentina.

Chromatographic analysis. At an appropriate time, aliquots of 100 µL were removed and then centrifuged. Then, 50 µL of the resulting supernatant was added to a solution of 450 µL of the corresponding mobile phase. Each sample was immediately stored at −18 °C until use. Upon removal of the last samples, the stored solutions were allowed to warm up to room temperature, and then the concentrations of compounds from the work solutions were monitored by MLC to determine the rate at which the parent compounds disappear.

Lipophilicity and solubility

The partition coefficient (log $P_{o/w}$) was determined as previously described[14] using the guide of the Organization for Economic Cooperation and Development (OECD) for determination with RP-HPLC[21]. The solubility was determined based on the saturation shake-flask method as previously described[14] and analyzed by MLC.

In vitro permeability

The artificial membrane was performed by immersion of a cellulose ester support (0.22 µm pore size, 25 mm diameter,

diffusion area 0.502 cm^2, GSWPO2500, Millipore®) for 10 min in a lipid phase (phosphatidylcholine 68–73%, phosphatidylethanolamine 7–10% and lysophosphatidylcholine <3%, Lipoid®) with 10% *n*-octanol. The studies were performed using Franz horizontal diffusion cells, with donor and receptor compartments of approximately 2.2 mL each. The receptor compartment was filled with PBS buffer and the donor containing a solution of test compound with buffer pH 5.5 (approx. 0.3 mg mL^{-1}); both cells were subjected to constant agitation and the temperature was maintained at 37 °C using a water circuit[22].

Samples (1 mL) were drawn via syringe from the center of the receptor compartment at the following time intervals: 15, 30, 45, 60, 75, 90, 105, 125, 145, 165, 185, 205, 225 and 245 min. The removed sample volume was immediately replaced with the same volume of fresh receptor medium (PBS; pH 7.4).

The cumulative quantity of drug (mg mL^{-1}) penetrating the unit surface area of the membrane was plotted versus time (min). Linear regression analyses were performed by calculating the corresponding coefficient of determination (R^2) to evaluate linearity, and slope (S) was used to calculate the apparent permeability coefficient (P_{app}) by applying Equation (1):

$$P_{app} : S \times \frac{1}{C_o}, \quad (1)$$

where C_o is the initial drug concentration in the donor solution. Results were expressed as the mean ± SD of three independent experiments.

Binding protein

The frozen human plasma (Section Stability studies) was slowly thawed in cold water and filtered over glass wool. The concentration of total protein (TP) and albumin (Alb) was determined with a commercially available kit (Proti 2, Weiner lab®, Boston, MA) according to instructions provided by the manufacturer. For all used samples, TP values (5.5–5.8 g dL^{-1}) and Alb (3.6–4.1 g dL^{-1}) were within the reference values. Work solutions of Allop and **2a**, **2b**, **2e** and **2f** (1.0×10^{-7} mol mL^{-1}) in phosphate buffer pH 7.4 (PBS) were prepared and 0.6 mL of each one of the studied compounds was incubated with 0.6 mL of human plasma at 37 °C for 10 min, each in triplicate. Ultrafiltration procedure was performed in a micropartition system (Centrifree® UF Device, No. 4104, Millipore) equipped with Ultracel YM-T membrane (cut-off 30 000 Da). To determine the adequate centrifugation time, different solutions of human plasma were centrifuged at 2000 rpm for 5, 10 and 15 min, respectively. The centrifugation time of 5 min was the appropriate one. This factor is of crucial importance because if the amount of ultrafiltrate exceeds significantly the 25% of the initial volume, some displacements in the binding equilibrium of the drug to protein may occur as a result of the protein concentration in the upper compartment.

In parallel, the adsorption of Allop, **2a**, **2b**, **2e** and **2f** to the filtration membrane was investigated. Recoveries were found between 97.5 ± 1.5% and 98.2 ± 1.7%. MLC was used as a analytical method in all assays.

XO assay

The reaction mixture (1 mL) contained Tris–HCl (50 mM, pH 7.7, which was prepared immediately before use), XO (3.3 mU mL^{-1}) and solution of the test compound (Allop, **2a**–**f**; stock solution: 3.5×10^{-7} mol mL^{-1}). After preincubation at 25 °C for 10 min, the reaction was initiated by the addition of the substrate solution (100 μM xanthine in Tris–HCl), and the uric acid formation was followed by measuring absorbance at 290 nm during 10 min. A control experiment (C) was carried out by replacing the test compound volume with the same amount of Tris–HCl. Different final concentrations (10, 25, 50, 75 and 100 μM) of the test compounds were assayed. Each study corresponds to three experiments.

The formation of uric acid versus time for C and for each of the test compounds (X) was evaluated at different concentrations. Then, the percentage of the enzymatic activity (slope of C versus time – slope of X versus time) was plotted against the concentration of X, and the IC_{50} for X was determined.

Results and discussion

Chemistry

There are different synthetic approaches to obtain *N*-acyloxymethyl derivatives[23]. Studies of Allop suggest coexistence of both, the NH-5, NH-1 and NH-5, NH-2 tautomers of the molecule in solution[24]. So, Allop has three H's available (NH-1, NH-2 and NH-5), which could be substituted. NH-5 and NH-1 are more reactive, but at the same time strongly favor the formation of tautomers of Allop. For this reason, most derivatives studied in this work were substituted in position 1, and only *mono*-derivatives were evaluated.

Two different synthetic strategies were used to obtain the new derivatives (Scheme 1). Method (a) the intermediate 1-(hidroxymethyl)allopurinol (**1a**)[25] was made to react with acid chloride, which favored the formation of *N*-acyloxymethyl derivatives. By said method, the **2a**–**d** compounds were obtained (Scheme 1a). Method (b) Allop was made to react directly with the corresponding chloromethyl ester to obtain the **2e**, **f** compounds (Scheme 1b).

Although some of them have been previously reported (**2a**, **2c**, **2e** and **2f**)[16], these derivatives were obtained in this work with better yields and at a shorter reaction time.

The structures of six compounds were characterized by using high resolution mass spectrometry and spectroscopic methods (^{1}H-NMR, ^{13}C NMR, HSQC-DEPT and HMBC).

Analysis of the HMBC spectra of the compounds demonstrated that the substitution occurs at N1 position for the derivatives (**2a**–**e**), and at N2 position for the derivative (**2f**) and that for the first time, a correlation exists between H-1′ and C-7a and C-2′, while for **2f**, H-1′ is associated with the C-3 and C-2′. All the ^{1}H and ^{13}C NMR data of the compounds mentioned in the experimental section are in full agreement with the proposed structures. HSQC-DEPT and HMBC for Allop, **2c** and **2f** are shown in Figures S1–S3 (Supplementary material).

Analytical method

The need for a bioanalytical method is of primary importance during the development of a lead molecule to a preclinical candidate. Micellar liquid chromatography (MLC) is a simple, robust and well-established branch of high-performance liquid chromatography (HPLC) that can be used for the analysis of most drug materials in pharmaceutical preparations, serum and urine samples[26].

Chromatographic condition

The mobile phase selection was based on the resolution of the compounds and a suitable analysis time. In order to find the best composition that allows the simultaneous analysis of the six derivatives and Allop, each of them was injected in mobile phases that contained different SDS (M)/modifier (%, v/v) ratios at pH 3 to ensure that the compounds were in their neutral form. In the first experiment, mobile phases containing 0.05 M of SDS, and 1% of 1-propanol, 1-butanol or 1-pentanol (elution strength of these modifiers increased in the order 1-propanol, 1-buntanol, 1-

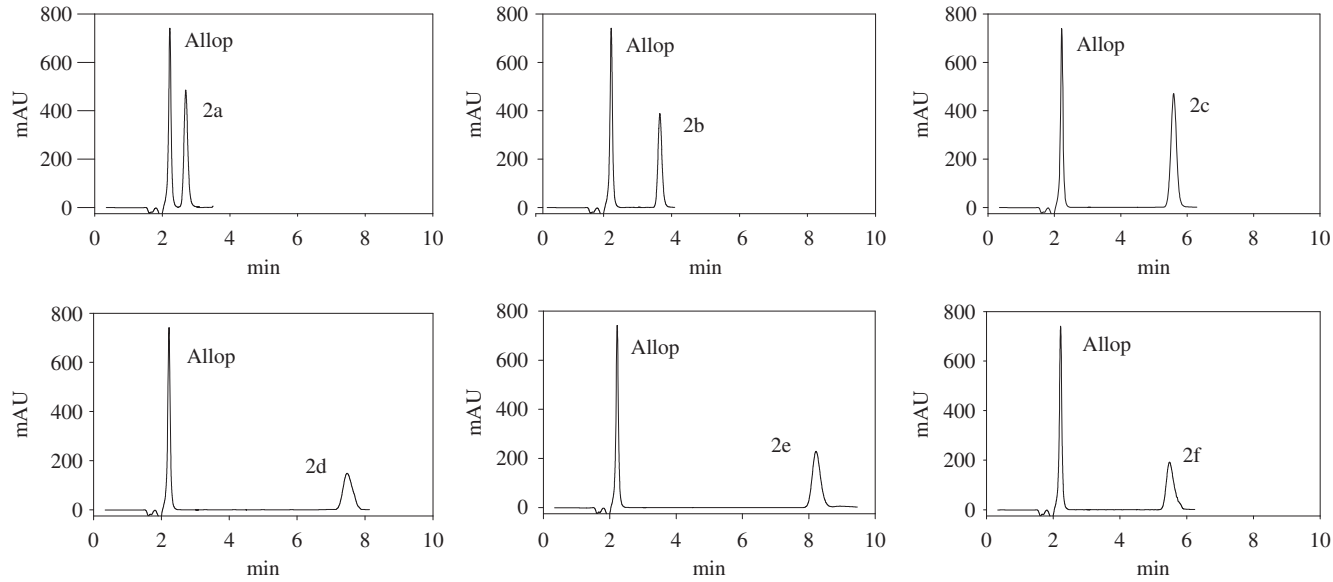

Figure 1. Chromatograms showing the separation of Allop, and its **2a–f** derivatives.

pentanol, according to the length of their chains) were employed. When 1-pentanol and 1-butanol were used, overlapping of some of the compounds took place, whereas 1-propanol allowed the complete resolution of all compounds. When SDS–1-propanol mobile phases were used and the maximum resolution–minimum analysis time criteria were applied, the mobile phase selected as being optimal was 0.1 M of SDS–7.5% (v/v) 1-propanol–NaH_2PO_4/H_3PO_4 (0.01 M, pH 3.0). Figure 1 shows chromatograms of Allop and its derivatives in the selected micellar mobile phase.

Validation

The ICH Harmonised tripartite Guideline: Validation of analytical procedures: text and methodology Q2 (R1) was chosen for the validation of the analytical methodology. This guideline presents a discussion of the parameters for consideration during the validation of the analytical procedures of drugs and pharmaceuticals in all kinds of matrices included as a part of registration applications submitted within the European Community, Japan and USA[27]. The parameters evaluated were specificity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness.

Specificity, linearity, limit of detection and limit of quantification. The chromatograms in Figure 1 show that there was no interference between Allop and their derivatives. Triplicate injections of six solutions of each compound at increasing concentration (ranging from 5×10^{-9} to 5×10^{-7} mol mL^{-1}) were carried out for the calibration in SDS and serum matrices. Both provided similar results. Table S1 (Supplementary material) lists the sensitivities (slopes of the calibration curves) and the intercepts. The linear regression coefficients were always >0.9998.

The LODs were calculated with the *3s criterion* (three times the standard deviation of the lowest concentration of the solution included in the calibration divided by the slope of the calibration curve) using a series of 10 solutions of each compound. The LOQs were determined using the *10s criterion*. The LODs and LOQs fell in the range of $0.17–0.4 \times 10^{-9}$ and $0.56–1.3 \times 10^{-9}$ mol mL^{-1}, respectively. The results are shown in Table S1 (Supplementary material).

Intra- and inter-day precision and accuracy. The intra- and inter-day precision at three different drug concentrations (7.5×10^{-9}, 6.0×10^{-8} and 3.5×10^{-7} mol mL^{-1}) was determined. The precision, expressed as relative standard deviations (RSDs), always was lower than 2.2% (see Table S2, Supplementary material).

The accuracy, expressed as an average of the percentage of recovery in *buffers* and all matrices, showed satisfactory recoveries (between 97% and 103%), and no significant deviations for all the studied compounds were observed. The parameters obtained are shown in Table S2 (Supplementary material).

Robustness. The robustness of the method was examined by replicate injections ($n = 6$) of a standard solution of each compound at 6×10^{-8} mol mL^{-1} with slight modifications to the chromatographic parameters (surfactant concentration, percentage of 1-propanol, pH and flow rate). The RSD obtained changing the retention time, peak area and resolutions were calculated, and were determined to be less than 2.0 (Table S3, Supplementary material). In conclusion, variations in all the studied parameters did not have a significant effect on retention time, peak area or resolution.

These results showed that the developed MLC method was suitable for the analysis of these compounds in buffer solutions and different matrices. Therefore, the procedure developed herein can be used for the quality control, routine analyses and pharmacokinetic studies of all the Allop derivatives.

Physicochemical properties

Acid dissociation constants (pK_a)

Meloun and Bordovská[28] evaluated the accuracy of the pK_a data generated by Advanced Chemistry Design (ACD/pK_a) packages, and concluded that this software provided the most accurate pK_a prediction. Therefore, Allop derivatives (**2a–f**) were evaluated using *in silico* method. Considering that Allop and the new derivatives exhibit a weak acid group (NH-5) responsible for the ionization, the pK_a is a useful physicochemical parameter to evaluate the extent of ionization of functional groups related to pH. All derivatives would be found in their neutral form at pH 7.4 (physiological). The results obtained are shown in Table 1.

Integrity

Stability studies are important parts of prodrug screening. The prodrugs need to have an acceptable chemical stability, but they

Table 1. Physicochemical properties of Allop and its derivatives.

		S_0			Lipinski rules (LR)					
	pK_a	25 °C	37 °C	mp (°C)	log P_{ow}	H-bond donor	H-bond acceptor	MW	PSA	NRB
Allop	9.3*	0.49 ± 0.05†	0.90 ± 0.04†	>350	−0.55‡	2	3	136.1	74.4	0
2a	9.6*	0.79 ± 0.09†	1.1 ± 0.2†	259¶	0.44	1	6	208.2	89.9	3
2b	9.6*	2.5 ± 0.1†	2.8 ± 0.3†	238¶	1.2	1	6	222.2	89.9	4
2c	9.6*	0.48 ± 0.03†	0.7 ± 0.1†	236¶	1.9	1	6	236.2	89.9	5
2d	9.6*	0.16 ± 0.01†	0.24 ± 0.03†	230¶	2.8	1	6	250.3	89.9	6
2e	9.6*	1.1 ± 0.4†	1.5 ± 0.2†	196¶	2.0	1	6	250.3	89.9	4
2f	9.6*	1.8 ± 0.1†	2.9 ± 0.4†	189¶	1.7	1	6	250.3	89.9	4
LR					<5	≤5	≤10	<500	–	–
OP‡									<140	<10
Tox§					>3				<75	

pK_a, acid dissociation constant; S_0, intrinsic solubility; mp, melting point; log P_{ow}, lipophilicity; MW, molecular weight; PSA, polar surface area in Å^2; NRB, number of rotatable bonds.
*±(0.4) acid group.
†mg mL^{-1}
‡Optimal properties.
¶±(2).
§Odds rations for the appearance of toxicity.

Table 2. Indicative of stability for Allop and its new derivatives (**2a–f**).

Compounds	No degradation†		P_{app} (10^{-5} cm/s)	f_b %	XO IC_{50} (μM)	υ
Allop	SIF	Human* plasma	0.66 ± 0.01	Insignificant‡	1.6 ± 0.2	–
2a	0.03 ± 0.01† <6¶	0.31 ± 0.06† 223.6§	0.9 ± 0.2	23 ± 2	53 ± 1	0.52
2b	0.31 ± 0.01† 42.7¶	0.52 ± 0.03† 133.3§	1.3 ± 0.1	49 ± 2	75 ± 3	0.56
2c	1.10 ± 0.01† 85.4¶	2.3 ± 0.2† 29.6§	2.8 ± 0.4	Not determined by instability	75 ± 1	0.68
2d	3.8 ± 0.1† 100¶	4.6 ± 0.4† 15.0§	3.0 ± 0.9	Not determined by instability	85 ± 6	0.68
2e	0.05 ± 0.01† <9¶	0.11 ± 0.03† 630.1§	6.3 ± 0.9	30 ± 2	>100	1.2
2f	0.04 ± 0.01† <7¶	0.06 ± 0.02† 1155.2§	2.6 ± 0.3	29 ± 4	>100	1.2

P_{app}, apparent permeability coefficient; f_b (%), percentage of PP binding; XO IC_{50}, xanthine oxidase inhibitory activity; υ, Charton steric parameter.
*50%, 37 °C.
†k_{obs} 10^{-2} min^{-1}.
‡Elion et al.
¶% Degradation (37 °C, 3 h).
§$t_{½}$ (min).

also have to cleave to active drugs under certain enzymatic conditions.

Prodrugs intended for oral dosing are often tested for pH stability at pH physiological, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Plasma stability under enzymatic conditions is also investigated to predict the rate of conversion to the active compound[20]. Thus, in this study the inherent stability of derivatives under these conditions was determined using a validated MLC as the analytical method.

The regeneration of the parent drug from *N*-acyloxymethyl derivatives occurs via a two-step reaction: cleavage of the ester bond followed by a spontaneous and fast decomposition of the *N*-hydroxymethyl intermediate[16,23].

Buffers, SGF, SIF and human plasma. The Food and Drug Administration Guidance for Industry[29] concerning *in vivo* bioavailability and bioequivalence studies for immediate release solid oral dosage forms includes a discussion on gastrointestinal stability. The drug loss from the gastrointestinal tract takes place by intestinal permeation or by a degradation process in the gastrointestinal fluids prior to membrane absorption. Stability in the gastrointestinal tract may be confirmed by incubating the drug substance in gastric and intestinal fluids that are representative of *in vitro* drug exposure to these fluids. Table 2 shows the kinetic constants (k_{obs}, apparent pseudo first-order kinetics), percentages of degradation (%) and half-life times for Allop and **2a–f** compounds in SIF and human plasma.

Allop and the new derivatives were stable at 100% at pH 1.2 and SGF for 1 h, and 3 h at pH 6.8 (data not shown), but showed degradation in SIF. Those derivatives with linear substituents (**2a–d**) increased their instability as the length of the carbon chain increased. However, the derivatives with branched substituents (**2e–f**) showed a similar stability between them which, in turn, were comparable to **2a** derivative that has the shortest carbon chain.

In human plasma, the tendency of instability can be compared with what was observed in SIF, where the linear substituents were

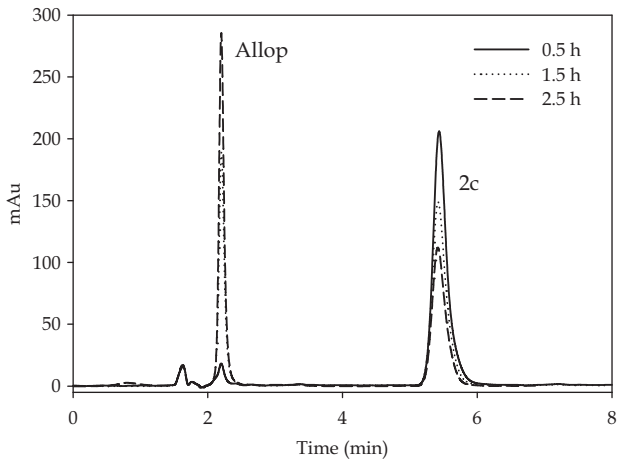

Figure 2. MLC chromatogram shows the retention time of **2c** and Allop in SIF, at different times.

the most unstable ones, while the branched substituents were the most stable compounds, showing a difference in this medium compared with **2a** derivative because the hydrolysis was caused by enzymes, so it is expected that the steric factor has the greatest impact.

On the basis of the results obtained, it is possible to conclude that all derivatives act as prodrugs since all of them regenerate the lead compound, whose anti-trypanosomal activity is widely recognized.

The analytical method used, MLC, proved to be a simple, rapid, precise and accurate method to study the stability of all new compounds. For example, Figure 2 shows the MLC chromatograms for **2c** in SIF medium at different times.

Lipophilicity and aqueous solubility

The partition coefficient (log $P_{o/w}$) of the new compounds were determined using RP-HPLC, where the value of chromatographic capacity factor (log k') is an indirect reliable descriptor of lipophilicity and can be directly related to the log $P_{o/w}$ value, which is regarded as a special case of the Collander equation[21]. This methodology has been already validated and previously used[14].

The results obtained (Table 1) show that the derivatives are all more lipophilic than the parent Allop; this would increase the absorption of the compounds by passive diffusion.

With regard to solubility, Allop has a very low solubility which is related to high crystal lattice energy (CLE). This property is directly associated with the mp[30]. The substitution of H at position 1 allows obtaining derivatives with a lower mp (lower CLE), so they are expected to have a higher aqueous solubility. Conversely, an increase in lipophilicity is associated with a low aqueous solubility. Thus, as a result of both characteristics (CLE and lipophilicity), it is possible to observe a varying behavior in the solubility of derivatives. This same behavior for various *N*-acyloxymethyl allopurinol derivatives was observed and explained by Bundgaard[16].

Other relevant properties

The oral administration is the most convenient, safest and least expensive way to deliver a drug; it is the route most often used[31]. During the 1990s almost 40% of the failure in clinical trials was attributed to poor absorption and pharmacokinetics. In response, pharmaceutical researchers began to focus on a better understanding to target selectivity, toxicological and physicochemical (pharmacokinetics related) properties of new chemical entities (NCE). Analysis of the structures of orally administered drugs, and of drug candidates, headed by Lipinski et al. led to the establishment of the ''rule of five''[32], and nowadays is the most widely used and accepted method.

Other parameters such as polar surface area (PSA) and molecular rigidity as indicated by the number of rotatable bonds (NRB) have also been associated with drug ability of NCEs[33].

Table 1 shows these parameters of each compound, as well as the limits for every one property. It could be observed that all of them exhibited acceptable characteristics. If any of these parameters are out of range, a poor absorption or permeability is possible.

To analyze the relationship between the physiochemical properties and toxicity[34], different descriptors were obtained from the *in vivo* analysis of about 250 preclinical candidates. PSA and theoretical log *P* (CLOGP) were the most closely related to the observation of toxicity. Their thresholds were set at a PSA < 75 Å^2 and clog $P > 3$. Table 1 shows that all derivatives exhibited acceptable characteristics.

In vitro permeability

One property of particular importance is the ability of drugs to cross biological membranes. The biological permeability of a drug shapes its pharmacokinetic profile in the body, affecting its absorption, distribution and elimination. The *in vitro* non-cell-based test represents an interesting alternative to the use of animal tissues or cells because of its simplicity, fast membrane preparation and high tolerance to a wider pH range (for better coverage of intestine pH range). In addition, it is cheap and shows comparable results to Caco-2 permeability[35,36].

The passive diffusion was evaluated using Franz horizontal diffusion cells (the method developed and validated by Delrivo[22]) at 37 °C with an artificial membrane constructed by lipids and *n*-octanol on a hydrophilic membrane support and cells (pH 5.5_{donor} – pH $7.4_{acceptor}$).The ideal pH used for *in vitro* screening ought to reflect the *in vivo* pH conditions[35].

The apparent permeability coefficient value (P_{app}) was determined by this assay (Table 2). All Allop derivatives increased the Papp with respect to Allop (up to nine times), which provides an important pharmacokinetic advantage. Besides lipophilicity, there are different physicochemical properties that influence oral bioavailability[37]. So, we determined that although **2d** derivative had the highest lipophilicity, **2e** compound showed greater Papp because of the increase in molecular rigidity (indicated by a lower NRB) which is a positive aspect for bioavailability[37].

Binding protein

The binding protein plays an important role in the pharmacokinetics and pharmacodynamics of a drug. Only the unbound drug is thought to be able to diffuse across membranes and to interact with an effector site to produce a therapeutic response[38]. In addition, considering that plasma protein (PP) acts as a reservoir of the bound drug, only the unbound drug is available for metabolization and elimination. It is known that Allop is negligibly bound to PP[39], so the design of new derivatives of Allop with an increased binding to PP would be of great therapeutic utility since it would increase the half-life time of the drug.

The ultrafiltration is undoubtedly the most widely used method because of its simplicity and general applicability to many different systems[38,40]. This method was employed to separate the unbound Allop and derivatives from the PP-Allop and

PP-derivatives complex by means of the Centrifree® device. The ultrafiltrate was analyzed by MLC.

The binding between PP and Allop, **2a, 2b, 2e** and **2f** were studied. Compounds **2c** and **2d** derivatives showed significant degradation in plasma in the time required to achieve balance[40]. The results obtained are summarized in Table 2.

The analysis of **2a**, **2e** and **2f** derivatives (excluding the **2b** derivative) demonstrated that there is a relationship between the bound fraction of PP and their lipophilicity (r^2: 0993), but it is well-known that lipophilicity is one, but not usually the only descriptor that can influence the PP binding[31,41].

XO binding

Allop is converted by XO into oxypurinol, which is detected in the circulation within 15 min of Allop administration[42]. (Oxypurinol is not a substrate for HGPRT and has no anti-trypanosomal activity[6].)

It was therefore appropriate to evaluate the affinity that these new derivatives have for this enzyme. To do this, IC_{50} was determined (concentration of the compound whose enzymatic activity was inhibited to 50%) as a measure of affinity. The experimental procedure employed was adapted from that reported by Gupta et al.[43] In this procedure, the enzyme (XO) with its natural substrate, xanthine, was incubated and the uric acid formation (regarded as an enzymatic activity of 100%) was quantified by UV spectrophotometry. Then, increasing concentrations up to 100 μM of the compound to be analyzed (Allop and **2a**–**f** derivatives) were added and the effect of said aggregates, that is the IC_{50} variation, was assessed. The values obtained from the analysis of three independent observations are shown in Table 2.

As can be seen, Allop has a high affinity for the enzyme (a concentration of only 1.5 μM causes 50% inhibition of this enzyme). However, the derivatives did not show that affinity. This can be partly related to the size of the substituent, so it is possible to use the Charton steric parameter (υ), which is based on the intrinsic size of the substituent and defined by the difference between the mean of Van der Waals radius of the substituent and the Van der Waals radius of hydrogen atom[44]. Although there is no directly proportional relationship between IC_{50} and υ, it can be seen that the affinity of the derivative for the enzyme ($>IC_{50}$) decreases as the size of the substituent increases, which can be associated with the steric hindrance of the compound. On the basis of the results obtained, it is possible to expect that the new derivatives protect Allop against the enzyme that metabolizes it. However, as a function of time, **2a**–**f** derivatives regenerate Allop, which would be a reservoir of the active compound.

Conclusions

Six prodrugs of Allop were obtained; two of these were not previously reported. In addition, they were all synthesized by new methodologies that meant a shorter reaction time and higher yields.

All compounds were thoroughly evaluated and those physicochemical properties that have a direct impact on the bioavailability of the drugs were analyzed. The results showed that all derivatives had a better physicochemical profile than Allop, met Lipinski's rules and had suitable characteristics of PSA and NRB.

Additionally, it should be noted that these derivatives act as prodrugs that regenerate Allop, the active compound, and the speed at which they do so (a $t_{1/2}$ ranging from 30 min to 19 h approximately) varies according to the substituent. This offers a wide range of possibilities as the needs may require.

All Allop derivatives increased the P_{app} with respect to Allop (up to nine times), and exhibited greater binding to PPs, and a lower affinity for XO. All these details provide an important pharmacokinetic advantage.

On the basis of these results, it is clear that these prodrugs of Allop, with better pharmacokinetic properties than the lead compound, would be a great alternative treatment for Chagas disease since they generate *in vivo* the compound with recognized activity.

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Declaration of interest

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Supplementary material available online
Supplementary Figures S1–S3 and Tables S1–S3