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Synthesis and biological activity of cyclopropyl Δ7-dafachronic acids as DAF-12 receptor ligands†

Valentina Mancino,[a,b] Giada Ceccarelli,[a] Andrea Carotti,[a] Laura Goracci,[c] Roccaldo Sardella,[a] Daniela Passeri,[b] Roberto Pellicciari[b] and Antimo Gioiello *[a]

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The four cyclopropyl stereoisomers of Δ7-dafachronic acids were prepared from the bile acid hyodeoxycholic acid and employed as chemical tools to exploit the importance of the orientation and spatial disposition of the carboxyl tail and the C25-methyl group for the binding at the DAF-12 receptor. The synthesis route was based on (a) Walden inversion and stereoselective PtO_2-hydrogenation to convert the L-shaped 5β-cholanoid scaffold into the planar 5α-sterol intermediate; (b) two-carbon homologation of the side chain by Wittig and cyclopropanation reaction; and (c) formation of the 3-keto group and Δ7 double bond. The synthesized isomers were isolated and tested for their activity as DAF-12 ligands by AlphaScreen assays. Results showed a significant loss of potency and efficacy for all the four stereoisomers when compared to the parent endogenous ligand. Computational analysis has evidenced the configurational and conformational arrangement of both the carboxylic and the C25-methyl group of dafachronic acids as key structural determinants for DAF-12 binding and activation.

Introduction

Dafachronic acids (DAs, **1–4**) (Fig. 1A) are C27 acidic steroids that serve as hormonal ligands for the nuclear hormone receptor DAF-12 ('DAuer larva formation-12'), a transcription factor regulating the life cycle fate of nematodes.[1] Acting as a molecular switch, the liganded DAF-12 promotes larval reproductive development, whereas the unliganded receptor directs towards a long-lived, alternate third larval stage, also called the dauer diapause, in which the nematode exhibits more stress resistance, an extended larval survival and, interestingly, an increased life span.[2] Remarkably, DAs also activate parasitic DAF-12 orthologues inducing the recovery of the third stage of infective larvae (iL3) in parasitic nematodes, such as *S. stercoralis*, and hookworms.[3,4] These important findings have suggested potential applications of DAF-12 modulators as therapeutic agents for the treatment of parasitosis as disseminated strongyloidiasis.[5]

Over the last years, diverse routes for the synthesis of endogenous DAF-12 ligands **1–4** [6,7] have been developed facilitating their use as chemical tools to explore the druggability of the receptor especially in agriculture and veterinary. DAs (**1–4**) are characterized by a planar steroidal body, a double bond at the C4 or C7 position, a C-3-keto group, a seven-carbon side chain with a methyl group at the C25 position and a carboxylic

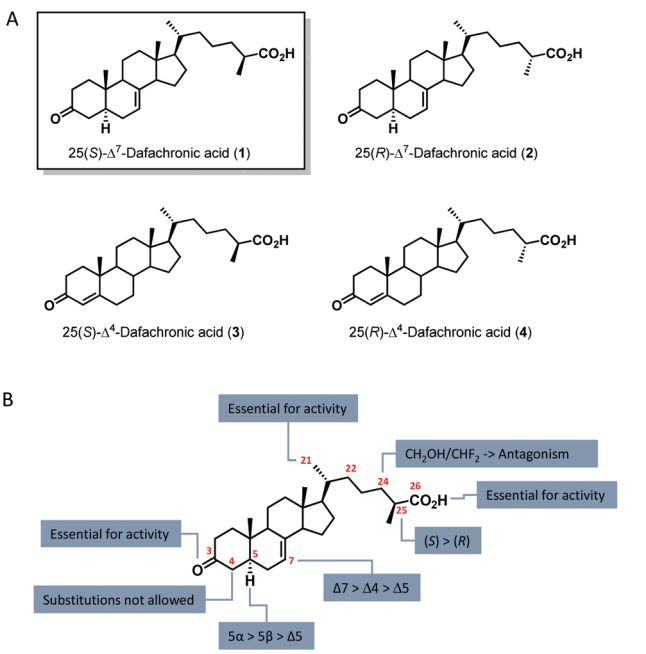

Fig. 1 (A) Structure of dafachronic acids (**1–4**). (B) Structure–activity relationships of dafachronic acid derivatives as DAF-12 ligands: key structural determinants and rank order of potency.

[a]*Department of Pharmaceutical Sciences, University of Perugia, Via del Liceo 1, 06123 Perugia, Italy. E-mail: antimo.gioiello@unipg.it*
[b]*TES Pharma S.r.l., Corso Vannucci 47, 06121 Perugia, Italy*
[c]*Department of Chemistry, Biology and Biotechnology, University of Perugia, Via dell'Elce di Sotto 8, 06123 Perugia, Italy*

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tail (Fig. 1A). Efforts in the structural modifications of the DA scaffold have been useful to define the structure–activity relationship (SAR) of steroidal DAF-12 modulators with the scope to enable the design and discovery of more effective compounds especially in terms of metabolic stability (Fig. 1B).[8–12]

In particular, it was shown that (a) 25(*S*)-diasteroisomers are more potent DAF-12 agonists than the corresponding (*R*)-epimers;[8] (b) 5α-steroids (*trans* A/B-ring junction) are better ligands than the corresponding 5β-analogs;[8] (c) the shift of the double bond to the C5 position and the replacement of the C20-methyl with a carbonyl group determines a significant loss of potency;[9,10a] (d) the side chain shortening has resulted in partial agonist compounds;[10a] (e) the introduction of a double bond at the C22 position, the insertion of a C24-difluomethylated group, or the replacement of the carboxylic moiety with a hydroxyl group yielded derivatives with an antagonistic profile;[10,11] (f) substitutions at the C4 position are not allowed.[12] It is worth noting that none of the DA derivatives was more potent than the natural ligands **1–4**, with the Δ7-(*S*)-DA (**1**) being the most active stereoisomer in both *in vitro* and *in vivo* appraisals.

Herein, we report the synthesis of 24,25-cyclopropyl DA derivatives **5–8** (Fig. 2) with the aim to evaluate the effect of the structural constraint and property of the cyclopropyl moiety in the activation of the DAF-12 receptor. Cyclopropyl analogues can indeed bind selectively to the target receptor as the result of a special arrangement and dedicated interactions, superimposed on the bioactive conformation. We also show results from biological screening experiments against the DAF-12 of *S. Stercolaris* (*ss*DAF-12) and provide computational modeling analysis to rationalize the observed DAF-12 activity.

Results and discussion

Design

Since the discovery of DAs **1–4**, several semisynthetic DA derivatives have been reported,[8–12] showing that a C-3 keto group and an unsaturated double bond at C-7 or C-4 are required for an efficient DAF-12 activation. As mentioned, the C25(*S*) diastereoisomers of both Δ4 and Δ7 DAs (**1** and **3**) are more active than the corresponding *R*-isomers (**2** and **4**), indicating that the configuration of the methyl group affects either ligand binding or complex conformation.[8] Moreover, it was shown that the lack of the C25-methyl decreased ligand affinity resulting in a less potent steroid.[10b]

Based on these findings, we sought to synthesize 24,25-methylen-Δ7-dafachronic acids (**5–8**) as DAF-12 ligands. The frequent appearance of the cyclopropyl ring in bioactive compounds and drugs is due to the influence that this moiety plays on the properties of the compounds containing it.[13] Indeed, the cyclopropyl ring addresses multiple roadblocks that can occur during medicinal chemistry such as enhancing potency, reducing off-target effects, increasing metabolic stability and permeability, decreasing plasma clearance, altering pK_a, and contributing to an entropically more favorable binding to the receptor. Moreover, the insertion of a cyclopropane ring at the α-position of a functional group responsible of key interactions with the receptor ligand binding domain (LBD) has been an extensively used strategy to obtain constrained analogues and determine the relative bioactive conformation.[14–16] In our case, the presence of the cyclopropyl ring at the C24–C25 position will confer a greater rigidity to the DA side chain terminus, allowing to get insights into the importance of the configurational and conformational aspects of the carboxyl and C25-methyl group in the interaction with crucial residues of the DAF-12 LBD. This is due to the distinctive geometric, steric and electronic features of the cyclopropylic nucleus. Moreover, it can be expected to achieve metabolically stable derivatives upon replacement of the C25 methyl group with the cyclopropane ring. Indeed, proton abstraction from a cyclopropane ring would be more difficult from a methyl group that is needed for oxidative metabolism to occur.

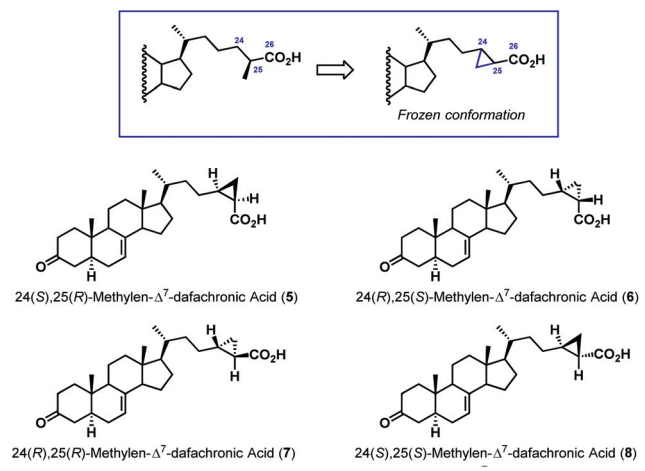


Fig. 2 Structure of the four isomers of 24,25-methylen-Δ^7-dafachronic acids (**5–8**).

Synthesis

In defining the best synthetic approach for the preparation of the four cyclopropyl diastereoisomers **5–8**, the knowledge acquired with the development of the divergent synthesis of DAs **1–4** was applied.[7] The synthesis pathway started from the natural bile acid hyodeoxycholic acid (HDCA, **9**) and was based on A/B-ring junction inversion, shift of the OH group from C6α- to C7β-position, side chain homologation, cyclopropanation reaction and generation of the Δ7 double bond, and C3-OH oxidation (Fig. 3).

The first stage of the synthesis was focused on modifications at the HDCA steroid nucleus (Scheme 1). Thus, the methyl ester of HDCA was treated with tosyl chloride (TsCl) in freshly distilled pyridine at room temperature to give the 3α,6α-ditosylated derivative in 94% yield. The methyl 3α,6α-ditosyloxy-5β-cholan-24-oate was refluxed in glacial AcOH in the presence of potassium acetate (AcOK) to provide the methyl 3β-acetoxychol-5-en-24-oate through the elimination of the C6-hydroxy group and the concurrent C3 Walden inversion.[17] Intermediate **11** was formed together with the

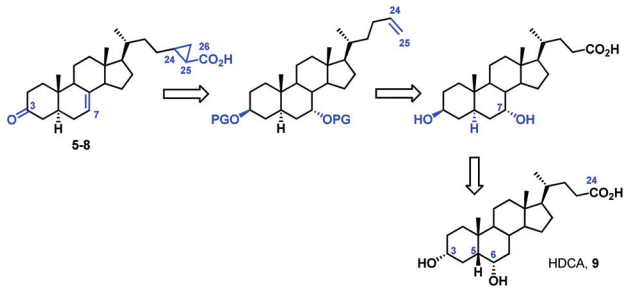
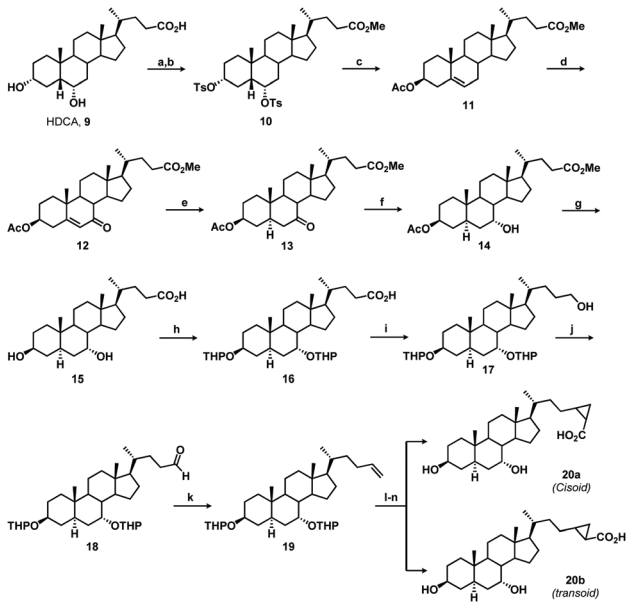


Fig. 3 Retrosynthetic approach to cyclopropyl dafachronic acids **5–8**. PG = protecting group.



Scheme 1 Synthesis of 3β,7α-dihydroxy-24,25-methylen-24-bishomo-5α-cholan-26-oic acid (**20a,b**) from hyodeoxycholic acid (**9**). Reagents and conditions: (a) *p*-TSA, MeOH, u.s.; (b) TsCl, dry pyridine; 94% from **9**; (c) AcOK, AcOH, reflux, 62%; (d) CrO_3, 3,5-dimethylpyrazole, CH_2Cl_2, −30 °C, then r.t., 65%; (e) H_2, PtO_2, 1.5 atm, AcOEt, 96%; (f) L-selectride, dry THF, −78 °C, then r.t.; (g) NaOH, MeOH, 72% from **13**; (h) 3,4-DHP, *p*-TSA, dioxane, >98%; (i) $LiAlH_4$, dry THF, 0 °C, then, r.t., 91%; (j) $(COCl)_2$, DMSO, Et_3N, dry CH_2Cl_2. −50 °C, 90%; (k) CH_3PPh_3Br, *t*-BuOK, dry THF, r.t., then reflux, 92%; (l) EDA, $Rh(OAc)_2$, dry CH_2Cl_2; (m) HCl, MeOH; (n) NaOH, MeOH, 75% from **19**.

methyl chol-3,5-dien-24-oate, whose formation was favored by the generation of a conjugated dienic system and by the trans-diaxial elimination of a water molecule from the C5–C6 position. The reaction showed a variable yield (21–62%) according to the batch size and concentration (data not shown). In particular, the yield resulted generally higher on small scale (up to 0.5 g) and in diluted conditions (0.2–0.3 M).

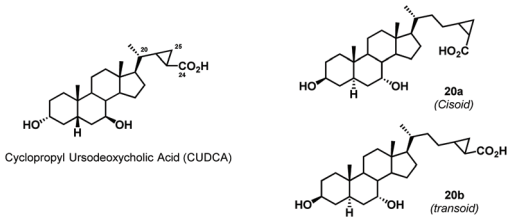
Intermediate **11** was next subjected to regioselective allylic oxidation by treatment with CrO_3 and 3,5-dimethylpyrazole in dry CH_2Cl_2 at room temperature to furnish **12** in 65% yield after flash chromatography. The C5–C6 double bond was reduced by stereoselective hydrogenation in the presence of PtO_2 to give the 5α-derivative **13** in excellent yield (96%). The reduction was performed also using Pd/C in THF, but the reaction proceeded considerably slowly. The subsequent stereoselective reduction of the C7 carbonyl group with L-selectride® in dry THF at −78 °C, provided the 7α-hydroxy intermediate, that was hydrolyzed under alkaline conditions (NaOH/MeOH) furnishing the acid **15** in 72% yield.

A suspension of **15** and catalytic *p*-TSA in dioxane at room temperature was reacted with 3,4-dihydro-2*H*-pirane to obtain the 3β,7α-protected acid **16** (>98%) to be converted into the corresponding aldehyde. As attempts for the direct reduction were not successful (data not shown), we were forced to first reduce the acid to alcohol using $LiAlH_4$ in freshly distilled THF at room temperature (91% yield) and then to oxidize the alcohol by Swern reagent (90% yield). The following Wittig reaction with methyltriphenylphoshonium bromide in refluxing THF using *t*-BuOK as a base afforded the alkene **19** in 92% yield after chromatography.

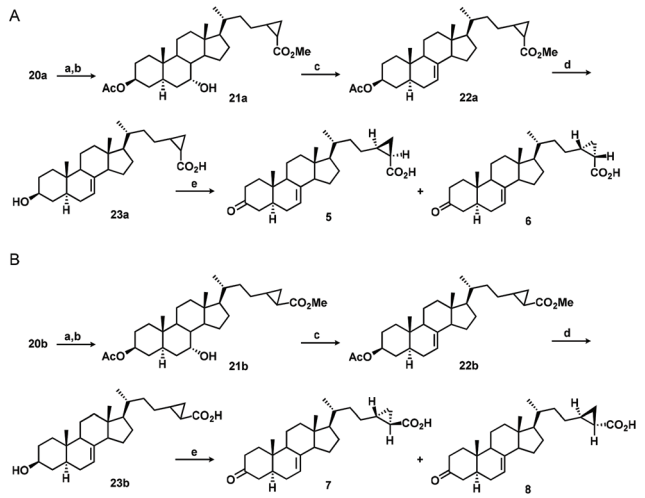
The cyclopropanation on the C24–C25 double bond was realized by slow addition of ethyl diazoacetate (EDA) to a solution of the alkene in dry CH_2Cl_2 at room temperature using dirhodium(II) tetraacetate ($Rh_2(OAc)_4$) as the catalyst.[18] As expected, the reaction furnished a mixture composed by the four cyclopropyl diastereoisomers with an overall yield of 92%. Finally, acidic (HCl/MeOH) and alkaline (NaOH/MeOH) hydrolysis provided isomers **20a** and **20b** in 6 : 4 ratio as determined by reverse phase HPLC analysis (Fig. 1S, ESI†). Crude purification by silica gel flash-chromatography allowed to isolate the two couples of isomers [R_f1 (**20a**), R_f2 (**20b**)], obtained in 45% and 30% isolated yield, respectively. At this stage, the stereochemistry of the two couple of isomers was hypothesized by NMR analysis, in analogy to previous works.[16,19] In cyclopropyl ursodeoxycholic acid (CUDCA), C-NMR analysis showed that signals related to the C24 carboxyl group, the C25 methylene and the C20 were more shielded for cisoid compounds than their respective transoid analogues (Table 1). The ^{13}C-NMR spectra of the two isomeric mixtures **20a** and **20b** revealed a similar tendency allowing us to speculate that compounds with the higher TLC retention factor (R_f) were the cisoids, while the couple of stereoisomers with a lower R_f were the transoid ones.

In the last part of the synthesis, the two couples of isomers **20a** and **20b** were elaborated separately using the same experimental protocol (Scheme 2). Therefore, the corresponding methyl ester of **20a,b** was selectively protected at the C3 position by treating with Ac_2O in presence of $NaHCO_3$ in refluxing THF, to afford the desired compounds **21a,b** in nearly quantitative yield (90%). These were treated with $SOCl_2$ in dry pyridine at room temperature to obtain the methyl-3β-acetoxy-24,25-methylen-Δ^7-DA derivatives **22a** and **22b** in 65% and 60% yield, respectively, after purification by flash chromatography on silica gel. Finally, the alkaline hydrolysis (NaOH/MeOH) and the treatment of the corresponding acids with Jones reagent in acetone furnished the desired cisoid **5–6** and transoid products **7–8** as mixtures of isomers, in high yields.

Table 1 Chemical shifts related to C20, C24 and C25 of 22,23-cyclopropyl ursodeoxycholic acids compared with chemical shift of C23, C26 e C27 of intermediates **20a,b**



	CUDCA			Intermediates **20a,b**		
	C20	C24	C25	C23	C26	C27
Cisoid	34.8	175.8	16.3	23.04	176.75	13.71
	31.8	176.1	12.0	23.18		13.98
Transoid	39.7	177.9	17.5	24.41	178.13	16.38
	39.4	177.3	12.7	24.61		15.83



Scheme 2 Synthesis of 24,25-metilen-Δ^7-DA (**5–8**) from intermediates **20a,b**. Reagents and conditions: (a) *p*-TSA, MeOH, u.s.; (b) Ac_2O, $NaHCO_3$, THF, 91% from **20a,b**; (c) $SOCl_2$, pyridine, 65% (**22a**) 60% (**22b**); (d) NaOH, MeOH, quantitative yield. (e) (i) Jones regent, acetone, 0 °C to r.t., 85%. (ii) HPLC purification.

In spite of their diastereomeric nature, compounds **5–8** failed to be separated on a conventional octadecylsilica-based achiral stationary phase, although it should in principle be conceptually possible. Therefore, compounds **5–8** were isolated using research-type non commercial *Cinchona* alkaloyd-based anion exchanger type chiral stationary phase.[20] The chemical integrity of the four isolated diastereoisomers was checked *via* HPLC, and the relative stereochemical assignment was determined as we previously reported.[20]

In vitro DAF-12 activity and molecular modeling

Pure cyclopropyl isomers **5–8** were tested along with 25(*S*)-Δ^7-DA (**1**) as reference control for their ability to bind and activate DAF-12 receptor using AlphaScreen assays (Table 2).

Table 2 DAF-12 activities of 25(*S*)-Δ7-DA (1) and cyclopropyl constrained analogs **5–8** [a]

	DAF-12 activity	
Compound	EC_{50} [a] (μM)	Efficacy [b] (%)
25(*S*)-Δ^7-DA (**1**)	0.011 ± 0.005	100
Cisoid (*S*,*R*) (**5**)	2.4 ± 0.6	50
Cisoid (*R*,*S*) (**6**)	8.0 ± 1.3	41
Transoid (*R*,*R*) (**7**)	4.0 ± 0.8	6
Transoid (*S*,*S*) (**8**)	11.4 ± 1.5	7

[a] Data represent EC_{50} mean values ± SD of at least three independent experiments. [b] % of efficacy was calculated at E_{max} of each curve considering the E_{max} of 1 as 100% value.

Unexpectedly, results disclosed a significant loss of activity for all the stereoisomers with derivative **5** being the most potent with an EC_{50} of 2.4 μM.

Furthermore, none of the derivatives reached the efficacy of **1**. In particular, cisoid compounds **5–6** were partial agonists while the corresponding transoid cyclopropyl analogs **7** and **8** failed to activate the receptor. Compound **7** was also tested in antagonist mode using 24-hydroxy-4-cholen-3-one early indicated as micromolar DAF-12 antagonist.[10] Data showed that compound **7** was able to displace the coactivator recruited by the agonist **1** suggesting an antagonist effect on the DAF-12 receptor. In particular, the dose response experiment using derivative **7** resulted an IC_{50} value of 25 μM while in the same experiment the IC_{50} value of 24-hydroxy-4-cholen-3-one was 3 μM.

In order to rationalize compound potency, molecular docking studies were carried out to evaluate the binding mode of **5–8** in the *ss*DAF-12 LBD (Fig. 4). In all cases, the steroid nucleus maintained the same arrangement within the receptor pocket, while the side chain showed a different arrangement. Indeed, although the carboxyl still formed H-bonds with the R599 and T613 residues, the different folding of the carbon chain caused a variation in the length and distance of these interactions.

The binding modes analysis highlighted that derivative **8** was the only one of the series that lost the H-bond interaction with T613. However, the docking score of **8** did not reflect this difference showing a value (−11.59) similar to the ones recorded for the other isomers (−11.38, −11.39 and −11.27 for **5**, **6** and **7**, respectively). Trying to understand the causes for the loss of activity observed for the cyclopropyl **5–8** with respect to **1**, a conformational analysis was performed to identify the global minima energy of each investigated compound. This value was instrumental to understand the energy spent by the ligand to assume the putative bioactive conformation inside the DAF-12 binding site. Interestingly, the bioactive conformation of **1** corresponded to its minima energy conformation, while all the cyclopropyl constrained DAs resulted with a less favorable energy profile. Indeed, compound **8** is 12.51 kJ mol^{-1} far from its energy minima, while the rest of the ligands gave slightly better results (7.82, 10.34 and

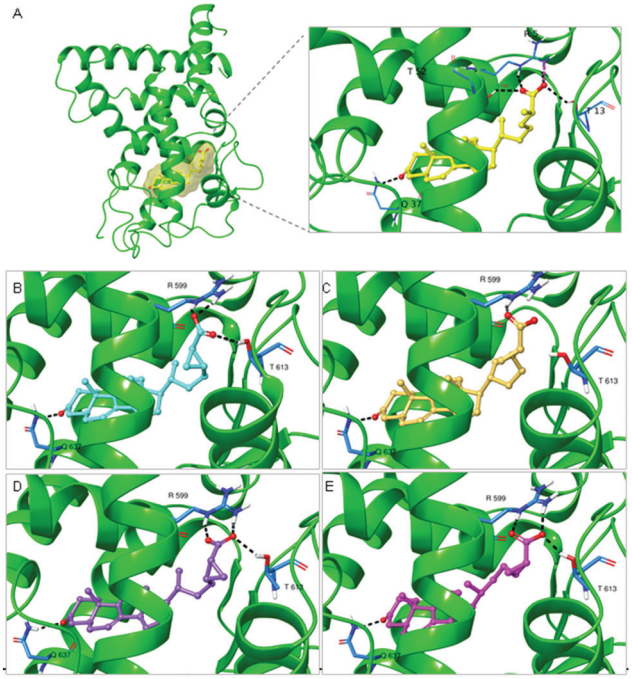


Fig. 4 (A) Binding mode of Δ7-(*S*)-DA (**1**) (yellow ball and sticks) observed in docking analysis using the 3GYU pdb code. (B–E) Docking results of **5–8** in the *ss*DAF-12 LBD: 24(*S*),25(*R*)-methylen-Δ7-dafachronic acid (**5**) (B); 24(*R*),25(*S*)-methylen-Δ7-dafacronic acid (**6**) (C); 24(*R*),25(*R*)-methylen-dafachronic acid (**7**) (D); 24(*S*),25(*S*)-methylen-Δ7-dafacronic acid (**8**) (E).

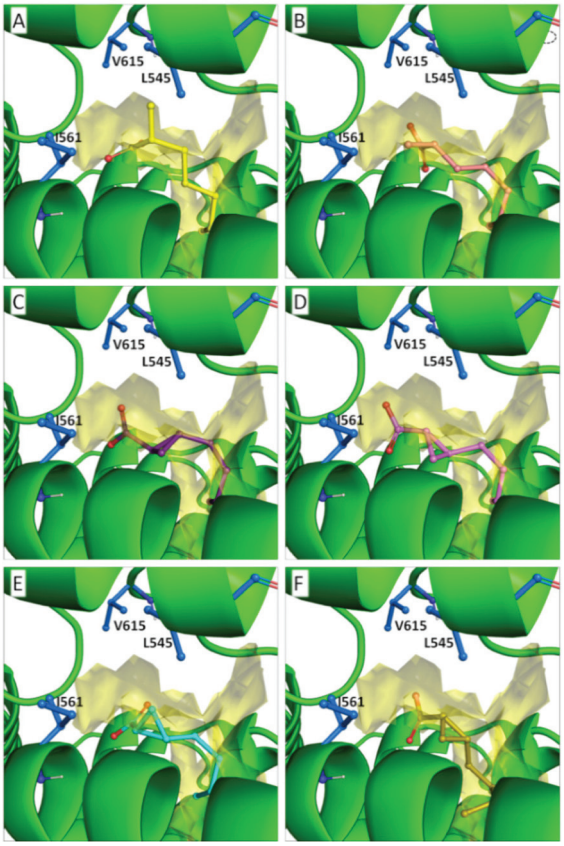


Fig. 5 Visualization of the CRY GRID MIF calculated in 3GYU pdb code at −1 kcal mol^{-1} and the docked poses from Fig. 3. (A) Δ7-(*R*)-DA (**2**); (B) Δ7-(*S*)-DA (**1**); (C) 24(*S*),25(*R*)-methylen-Δ7-dafachronic acid (**5**); (D) 24(*R*),25(*S*)-methylen-Δ7-dafacronic acid (**6**); (E) 24(*R*),25(*R*)-methylen-dafachronic acid (**7**); (F) 24(*S*),25(*S*)-methylen-Δ7-dafacronic acid (**8**).

9.66 kJ mol^{-1} for **5**, **6** and **7**, respectively) but still quite far from the most favored conformational energy value. It is worth noting that the trend of the conformational energy value calculated reflects the experimental DAF-12 activity observed. In conclusion, *in silico* analyses highlighted that the cyclopropyl constraint still allowed to keep the H-bond interactions with Q637, R599 and T613, but all of them spend a high conformational energy cost that may explain the observed drop in activity.

A further analysis was performed to investigate the possible role of the methyl group in **1** and **2** interacting with *ss*DAF-12 (Fig. 5). We hypothesized that its replacement with the cyclopropyl group could be also partially responsible of the decreased activity. To this aim, the hydrophobic regions in the protein cavities were evaluated in terms of GRID Molecular Interaction Fields (MIFs)[21] using the CRY probe, which simulates hydrophobic interactions, including entropic energy terms.[22] The CRY MIFs were visualized in the docking results (Fig. 5). This study highlighted a hydrophobic region generated by V615, L454 and I561, which is almost overlapped with the area including R599 and T613 residues, devoted to establish the H-bonds with the carboxyl moiety in the tested ligands. According to our docking results, both stereoisomers **1** and **2** are able to orient the methyl group into the CRY MIFs (Fig. 5A and B). On the contrary, the introduction of the cyclopropyl moiety in **5–8** hampers this hydrophobic interaction (Fig. 5C–F). In the past, the effect of the replacement of a hydrogen atom with a methyl group in protein binding was deeply studied by performing literature analysis in more than 2000 cases.[23] As a result, Leung and co-workers noticed that an activity boost of a factor of ≥10 occurred with an 8% frequency, while a 100-fold boost occurred as a 1 in 200 event. Therefore, in our study a cooperative effect including the conformational constraint and the lack of the methyl group might be responsible for the lower activity.

Conclusions

The high incidence of nematode parasitism in developing countries and the associated problems make the availability of adequate anthelmintic treatments necessary. Unfortunately, the available treatments are not fully effective because of the onset of resistance mechanisms and the high criticality of disseminated strongyloidiasis. The implication of the DAF-12 receptor in the regulation of parasitic life cycles and infective stages brought to light the opportunity to develop new

approaches for the treatment of parasitic diseases by using DAF-12 ligands.

The scope of this work was to get further insights into the role of the DA side chain terminal, and in particular, of the C26-carboxyl and C25-methyl group in the activation of the DAF-12 receptor. To this aim, we have synthesized the four stereoisomers of DA **5–8** characterized by the presence of the cyclopropyl ring at the α-position of the carboxylic acid. While this modification imposes a certain rigidity influencing compound binding at the receptor, the presence of the cyclopropane can provide metabolically stable DA derivatives and improve physico-chemical properties as water solubility.

The synthesis of the four stereoisomers **5–8** was realized from the bile acid hyodeoxycholic acid. Pure compounds **5–8** were prepared in 19 steps and evaluated for their ability to modulate the *ss*DAF-12 receptor, the most important parasitic receptor orthologue. Our results indicated that constraints at the side chain are detrimental for the potency of DAs likely due to the high conformational energy cost spent by the ligand to assume the putative bioactive conformation within the *ss*DAF-12 LBD. In addition, the lack of a methyl group pointing towards the hydrophobic region defined by V615, L545 and I561 could also play a role in making the binding less effective. Taken together, we can summarize that although the cyclopropyl-constrained DAs **5–8** are less active compared with the endogenous agonist **1**, we demonstrated the fundamental role of the DA side chain in the activation of the receptor demonstrating the importance of the configurational and conformational asset of both the carboxyl terminal and methyl group at the C25 position. Our study paves the way for the design of novel side chain-modified DA derivatives and furnishes a synthesis route to prepare useful tools to explore specific binding to DAF-12 receptor subtypes, especially to those lacking crystal structures.

Experimental

General methods

All the reagents were of analytical grade. All reactions were monitored by TLC, performed on aluminum backed silica plates 60-F254 (Merck). Spots on TLC were visualized by using UV lamp (254 nm) and by staining and warming with phosphomolybdate reagent (5% solution in EtOH). The purifications by flash chromatography were performed using silica gel F60 (Merck) (0.040–0.063 mm) or by Combi-flash using prepacked columns. ^{1}H NMR spectra were recorded at 200 or 400 MHz, ^{13}C NMR spectra were recorded at 100.6 MHz using the solvents indicated below. Chemical shifts are reported in ppm (parts per million). The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet, dddd, double double double doublet, t, triplet, dt, double triplet, qt, quartet triplet, bs, broad signal. Melting points were determined with an electrothermal apparatus and are uncorrected. The preparative and analytical HPLC measurements were made on a Shimadzu LC-20A Prominence equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector and a Rheodyne 7725i injector with a 20 μL stainless steel loop. A Lux Amylose-2 RP18 column (Phenomenex®, Torrance, CA, USA) (250 mm × 4.6 mm I.D.) was used as the analytical column. The column temperature was controlled through a Grace heather/chiller thermostat. Purity of synthesized compounds (>95%) was assessed by HPLC-HRMS.[20] Spectroscopic characterization of intermediates **12** and **15** are consistent with previously reported spectral data.[24,25]

Synthesis

Synthesis of Δ^7-dafachronic acids (5–8)

*Methyl 3β-acetoxy-chol-5-en-24-oate (**11**).* Intermediate **11** was prepared from 40 g HDCA (**9**) in refluxing AcOH following our previous reported protocol.[17] Isolated yield from HDCA (**9**): 56%. R_f (petroleum ether/AcOEt 98 : 2): 0.30. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 0.66 (3H, s, 18-CH_3), 0.90 (3H, d, J = 6.4 Hz, 21-CH_3), 0.99 (3H, s, 19-CH_3), 2.01 (3H, s, OCOCH_3), 3.64 (3H, s, COOCH_3), 4.57–4.61 (1H, m, 3-CH), 5.35 (1H, s, 6-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.81, 18.26, 19.27, 20.95, 21.42, 24.20, 27.70, 28.06, 30.96 (2×), 31.78 (2×), 35.32, 36.51, 36.92, 38.04, 39.61, 42.30, 49.90, 51.48, 55.68, 56.57, 73.94, 122.57, 139.57, 170.58, 174.78.

*Methyl 3α-acetoxy-7-keto-chol-5-en-24-oate (**12**).* To a suspension of CrO_3 (87.5 g, 0.88 mol) in dry CH_2Cl_2 (340 mL) at −40 °C, dry 3,5-dimethylpyrazole was added (84.0 g, 0.88 mol) and the mixture was stirred at −30 °C for 30 minutes. Intermediate **11** (20.9 g, 48.6 mmol) was added and the mixture was allowed to warm to room temperature and reacted for 2 d. The reaction mixture was filtered through a Celite pad, washing the filter with CH_2Cl_2, and evaporated to dryness. The crude was purified by flash chromatography using petroleum ether/AcOEt as eluent to furnish the desired product **12** as a white solid (14.1 g, 31.8 mmol, 65%). R_f (petroleum ether/AcOEt 85 : 15): 0.25. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.68 (3H, s, 18-CH_3), 1.86 (3H, d, J = 6.3 Hz, 21-CH_3), 1.20 (3H, s, 19-CH_3), 2.05 (3H, s, OCOCH_3), 2.20–2.25 (1H, m, 8-CH), 3.66 (3H, s, COOCH_3), 4.67–4.74 (1H, m, 3-CH), 5.70 (1H, s, 6-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.94, 17.21, 18.42, 21.10, 21.22, 26.21, 27.30, 28.35, 30.98 (2×), 35.22, 35.95, 37.69, 38.26, 38.56, 43.12, 45.33, 49.70, 49.87, 51.46, 54.41, 72.15, 126.65, 163.84, 170.26, 174.63, 201.77.

*Methyl 3β-acetoxy-7-keto-5α-cholan-24-oate (**13**).* To a solution of **12** (13.4 g, 30.1 mmol) in AcOEt (280 mL), PtO_2 was added (0.68 g, 3.0 mmol) and the resulting mixture was hydrogenated at 1.5 atm at room temperature for 16 h. The reaction product **13** was obtained by filtration through a Celite pad and evaporation of the solvent (12.8 g, 28.8 mmol, 96%). R_f (petroleum ether/AcOEt 8 : 2): 0.31. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.64 (3H, s, 18-CH_3), 0.91 (3H, d, J = 6.3 Hz, 21-CH_3), 1.10 (3H, s, 19-CH_3), 2.02 (3H, s, OCOCH_3), 2.15–2.25 (1H, m, 8-CH), 2.34 (2H, t, J = 6.3 Hz, 6-CH_2), 3.65 (3H, s, COOCH_3), 4.60–4.70 (1H, m, 3-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.67, 12.03, 18.33, 21.30, 21.73, 24.90, 27.05, 28.21, 30.94, 31.01, 33.78, 35.18,

35.78, 35.90, 38.61, 42.53, 45.81, 46.43, 48.81, 49.88, 51.46, 54.66, 54.89, 72.72, 170.46, 174.64, 211.41.

*Methyl 3β-acetoxy-7α-hydroxy-5α-cholan-24-oate (**14**).* To a solution of the 7-keto derivative **13** (12.8 g, 28.8 mmol) in freshly distilled THF (200 mL) at −78 °C, L-selectride® (43 mL, 43.0 mmol) was added dropwise in 30 min and the solution thus obtained was stirred at −78 °C for 50 min. Then, $NaHCO_3$ saturated solution (130 mL), 30% H_2O_2 (215 mL) and AcOEt (280 mL) were sequentially added dropwise. The mixture was stirred for 30 min at room temperature and the aqueous phase was extracted with AcOEt (2 × 300 mL). The combined organic layers were washed with brine (200 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness, to give the desired product **14** (14.1 g, 31.5 mmol) as a white solid, that was used for the next step without further purifications. R_f (petroleum ether/AcOEt 7 : 3): 0.26. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.64 (3H, s 18-CH_3), 0.80 (3H, s, 19-CH_3), 0.90 (3H, d, J = 6.4 Hz, 21-CH_3), 2.00 (3H, s, OCOCH_3), 2.15–2.25 (1H, m, 23-CH_2), 2.30–2.40 (1H, m, 23-CH_2), 3.65 (3H, s, COOCH_3), 3.81 (1H, bs, 7-CH), 4.66–4.72 (1H, m, 3-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.08, 11.77, 18.18, 20.87, 21.38, 23.53, 27.32, 28.01, 30.95 (2×), 33.52, 35.30, 35.48, 36.30, 36.42, 36.84, 39.35, 39.45, 42.60, 45.61, 50.42, 51.43, 55.71, 67.68, 73.52, 170.53, 174.67.

*3β,7α-Dihydroxy-5α-cholan-24-oic acid (**15**).* A solution of ester **14** (14.1 g, 31.5 mmol) in 5% NaOH in MeOH (125 mL) was stirred at room temperature overnight. The solvent was evaporated under reduced pressure, the resulted residue was dissolved in H_2O (200 mL) and washed with Et_2O (3 × 200 mL). The aqueous phase was acidified to pH = 4 with 3 N HCl and extracted with Et_2O (2 × 200 mL). The combined organic layers were washed with brine (200 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to obtain **15** as a white solid (8.9 g, 22.8 mmol, 72% from **13**). R_f ($CHCl_3$/MeOH 95 : 5): 0.27. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.54 (3H, s 18-CH_3), 0.69 (3H, s, 19-CH_3), 0.81 (3H, d, J = 6.4 Hz, 21-CH_3), 2.00–2.10 (1H, m, 23-CH_2), 2.13–2.17 (1H, m, 23-CH_2), 3.48–3.43 (1H, m, 3-CH), 3.68 (1H, bs, 7-CH), 3.80–3.90 (2H, s, 3,7-OH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 14.97, 15.59, 22.01, 24.82, 27.35, 34.76, 34.83, 34.88, 39.25, 39.36, 40.25, 40.60, 40.86, 41.13, 43.33, 43.39, 46.47, 49.60, 54.29, 59.67, 71.55, 74.64, 180.98.

*3β,7α-Ditetrahydropyranyloxy-5α-cholan-24-oic acid (**16**).* To a suspension of the acid **15** (1.5 g, 3.83 mmol) and *p*-toluensulfonic acid (0.11 g, 0.57 mmol) in 1,4-dioxane (40 mL), 3,4-dihydro-2*H*-pyran (3.5 mL, 38.3 mmol) was added dropwise in 10 min and the mixture was stirred at room temperature overnight. The mixture was poured into H_2O (80 mL) and extracted with AcOEt (5 × 50 mL). The organic phase was evaporated to dryness. The thick yellow oil was dissolved in MeOH (25 mL), treated with 10% aqueous NaOH (10 mL) and refluxed for 2 h. The solvent was evaporated under reduced pressure and the resulting residue was dissolved in H_2O (50 mL) and extracted with Et_2O (3 × 50 mL). The aqueous phase was acidified to pH = 5 with 1 N HCl and quickly extracted with AcOEt (3 × 50 mL). The combined organic layers were washed with H_2O (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness, providing the desired product **16** as a yellow oil (2.15 g, 3.83 mmol, quantitative). R_f (petroleum ether/AcOEt 8 : 2): 0.12. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.62 (3H, s, 18-CH_3), 0.78–0.79 (3H, m, 19-CH_3), 0.95 (3H, d, J = 6.2 Hz, 21-CH_3), 2.20–2.27 (2H, m, 23-CH_2), 2.30–2.40 (2H, m, 23-CH_2), 3.39–3.57 (2H, m, OCH_2-THP), 3.58–3.65 (1H, m, 3-CH), 3.70 (1H, bs, 7-CH), 3.85–3.93 (2H, m, OCH_2-THP), 4.57–4.60 (1H, m, OCHO-THP), 4.69–4.71 (1H, m, OCHO-THP).

*3β,7α-Ditetrahydropyranyloxy-5α-cholan-24-ol (**17**).* To a suspension of $LiAlH_4$ (1.74 g, 7.66 mmol) in dry THF (20 mL) at 0 °C, a solution of the acid **16** (2.15 g, 3.83 mmol) in dry THF (30 mL) was added dropwise. The solution obtained was stirred at room temperature for 18 hours, then AcOEt (15 mL) and MeOH (800 mL) were cautiously sequentially added dropwise and the mixture was poured into H_2O (150 mL) and extracted with AcOEt (5 × 60 mL). The organic phase was washed with brine (150 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to obtain the desired alcohol derivative **17** as a yellowish oil (1.91 g, 3.50 mmol, yield: 91%), used for the next step without further purifications. R_f (petroleum ether/AcOEt 8 : 2): 0.15. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.59 (3H, s, 18-CH_3), 0.75–0.76 (3H, m, 19-CH_3), 0.89 (3H, d, J = 4.8 Hz), 3.45–3.51 (2H, m, OCH_2-THP), 3.54–3.55 (1H, m, 3-CH), 3.67 (1H, bs, 7-CH), 3.82–3.88 (2H, m, OCH_2-THP), 4.54–4.56 (1H, m, OCHO-THP), 4.68–4.70 (1H, m, OCHO-THP).

*3β,7α-Ditetrahydropyranyloxy-5α-cholan-24-al (**18**).* To a solution of $(COCl)_2$ (0.40 mL, 4.55 mmol) in CH_2Cl_2 (5 mL) at −50 °C, DMSO (0.57 mL, 8.75 mmol) was added dropwise in 10 min. The mixture was then stirred for 10 min at −50 °C, prior adding dropwise a solution of the alcohol **17** (1.91 g, 3.50 mmol) in CH_2Cl_2 (10 mL). The mixture was stirred for 2 h at −50 °C. Et_3N (2.43 mL, 17.49 mmol) was added and the reaction was allowed to warm to room temperature in 4 h. The mixture was poured into 2 N aqueous KOH (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with H_2O (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness, to provide 1.72 g (3.16 mmol, 90%) of 3β,7α-ditetrahydropyranyloxy-5α-cholan-24-al (**18**) that was used for the next step without further purifications. R_f (petroleum ether/AcOEt 8 : 2): 0.39. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.63 (3H, s, 18-CH_3), 0.79–0.80 (3H, m, 19-CH_3), 0.91 (3H, d, J = 6.3, 21-CH_3), 2.30–2.38 (2H, m, 23-CH_2), 2.41–2.48 (2H, m, 23-CH_2), 3.46–3.50 (2H, m, OCH_2-THP), 3.57–3.67 (1H, m, 3-CH), 3.71 (1H, bs, 7-CH), 3.85–3.93 (2H, m, OCH_2-THP), 4.57–4.85 (1H, m, OCHO-THP), 4.71–4.74 (1H, m, OCHO-THP), 9.76 (1H, s, CHO).

*3β,7α-Ditetrahydropyranyloxy-24-homo-5α-chol-24-ene (**19**).* To a suspension of methyltriphenylphosphonium bromide (4.11 g, 4.31 mmol) in freshly distilled THF (30 mL), 1 M *t*-BuOK solution in THF (10.47 mL, 10.47 mmol) was added and the yellow mixture obtained was stirred overnight. A solution of the aldehyde **18** (1.9 g, 3.46 mmol) in dry THF (30 mL) was added to the suspension, which turned to orange, and the mixture obtained was refluxed for 3 h. The mixture was

allowed to cool to room temperature, diluted with *n*-hexane (100 mL) and H_2O/MeOH 1 : 1 (150 mL), and extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine (100 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude was purified by silica gel chromatography using petroleum ether/EtOAc (from only petroleum ether to petroleum ether/EtOAc 6 : 4) as eluent. Intermediate **19** was isolated as a white solid (1.73 g, 3.18 mmol, 92%). R_f (petroleum ether/AcOEt 8 : 2): 0.48. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.61 (3H, s, 18-CH_3), 0.78–0.79 (3H, m, 19-CH_3), 0.90 (3H, d, J = 6.4, 21-CH_3), 3.46–3.48 (2H, m, OCH_2-THP), 3.58–3.66 (1H, m, 3-C*H*), 3.70 (1H, bs, 7-C*H*), 3.83–3.92 (2H, m, OCH_2-THP), 4.55–4.58 (1H, m, OC*H*O-THP), 4.70–4.72 (1H, m, OC*H*O-THP), 4.95 (1H, d, J = 1.32 Hz, 25-C*H*), 4.97 (1H, d, J = 1.33 Hz, 25-C*H*), 5.73–5.83 (1H, m, 24-C*H*).

*Methyl 3β,7α-dihydroxy-24,25-methylen-24-bishomo-5α-cholan-26-oate (**20a,b**).* To a suspension of the intermediate **19** (1.5 g, 2.75 mmol) and $Rh_2(OAc)_4$ (49 mg, 0.11 mmol) in dry CH_2Cl_2 (40 mL), ethyl diazoacetate (EDA) (1.68 mL, 15.99 mmol) in dry CH_2Cl_2 (20 mL) was added using a syringe pump, with a rate of 1.5 mL min^{-1}. The mixture was reacted for 20 h, filtered over a Celite pad and evaporated to dryness. The crude was treated for 3 h with a 5% HCl solution in MeOH (30 mL). The solvent was removed under reduced pressure, then H_2O (100 mL) was added and the residue was extracted with $CHCl_3$ (3 × 70 mL). The combined organic layers were washed with H_2O (100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude was stirred with a 5% NaOH solution in refluxing MeOH (15 mL) overnight. The reaction mixture was concentrated *in vacuo*, diluted with H_2O (100 mL) and washed with Et_2O (50 mL). The aqueous phase was acidified to pH = 4 with 3 N HCl and extracted with EtOAc (5 × 100 mL). The combined organic layers were washed with H_2O (100 mL) and brine (100 mL), dried over Na_2SO_4 and evaporated to dryness. The crude isomeric mixture was purified by silica gel flash chromatography to obtain pure **20a** (400 mg) and **20b** (267 mg). **20a**: R_f (CH_2Cl_2/MeOH 9 : 1): 0.32. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.66 (3H, s, 18-CH_3), 0.81 (3H, s, 19-CH_3), 0.90 (3H, d, J = 6.4, 21-CH_3), 3.61–3.67 (1H, m, 3-C*H*), 3.83 (1H, bs, 7-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.65, 12.23, 13.71, 13.98, 18.98, 19.35, 22.13, 23.04, 23.18, 24.72, 28.06, 31.29, 35.53 (2×), 36.27, 36.71, 37.04, 37.59, 38.56, 39.53, 42.63, 42.62, 45.84, 49.63, 49.84, 50.50, 55.85, 67.97, 71.11, 176.75. **20b**: R_f (CH_2Cl_2/MeOH 9 : 1): 0.30. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.70 (3H, s, 18-CH_3), 0.84 (3H, s, 19-CH_3), 0.95 (3H, d, J = 6.4, 21-CH_3), 3.51–3.57 (1H, m, 3-C*H*), 3.77 (1H, bs, 7-C*H*). ^{13}C-NMR (MeOD + DMSO, 100.6 MHz) δ: 11.81, 12.41, 15.83, 16.38, 19.29, 20.83, 21.29, 22.19, 24.51, 29.34, 30.64, 32.26, 36.55, 36.72, 38.07, 38.26, 38.74, 40.18, 40.39, 41.01, 43.70, 47.09, 48.43, 49.70, 51.73, 57.51, 68.48, 71.82, 178.13.

*Methyl 3β-acetoxy-7α-hydroxy-24,25-methylen-24-bishomo-5α-cholan-26-oate (**21a**).* To a solution of the acid **20a** (107 mg, 0.25 mmol) in MeOH (20 mL), *para*-toluenesulfonic acid (20 mg, 0.10 mmol) was added and the solution was treated with ultrasound at room temperature for 90 min, then heated to reflux for 39 hours. The solvent was evaporated under reduced pressure, the residue was dissolved in H_2O (50 mL) and extracted with $CHCl_3$ (2 × 50 mL). The combined organic layers were washed with saturated $NaHCO_3$ (50 mL), H_2O (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness, to provide the desired methyl ester as a light yellow solid. To a solution of the ester in dry THF (8 mL), $NaHCO_3$ (360 mg, 4.32 mmol) and Ac_2O (0.41 mL, 4.32 mmol) were added and the mixture was refluxed overnight under magnetic stirring. The mixture was concentrated *in vacuo*, the residue was dissolved in H_2O (30 mL) and acidified to pH = 4 with 3 N HCl and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were washed with H_2O (30 mL) and brine (30 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure, obtaining the desired product **21a** as a yellowish solid (106 mg, 0.22 mmol, yield 91%). R_f (petroleum ether/EtOAc 8 : 2): 0.21. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 0.62–0.63 (3H, m, 18-CH_3), 0.80 (3H, s, 19-CH_3), 0.87 (3H, d, J = 6.13, 21-CH_3), 2.01 (3H, s, OCOCH_3), 3.65 (3H, s, COOCH_3), 3.80 (1H, bs, 7-C*H*), 4.60–4.70 (1H, m, 3-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.08, 11.73, 13.35, 17.81, 18.06, 18.71, 20.56, 21.40, 22.26, 22.48, 23.56, 27.31, 28.08, 33.51, 35.47, 35.60, 35.80, 36.22, 36.40, 36.83, 39.38, 42.54, 45.61, 50.41, 51.50, 55.83, 67.74, 73.54, 170.57, 173.48.

*Methyl 3β-acetoxy-7α-hydroxy-24,25-methylen-24-bishomo-5α-cholan-26-oate (**21b**).* Compound **21b** was obtained following the same procedure described for **21a**, starting from the acid **20b** (137 mg, 0.31 mmol) in 90% yield (138 mg, 0.28 mmol). R_f (petroleum ether/EtOAc 8 : 2): 0.19. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.64–0.65 (3H, m, 18-CH_3), 0.81 (3H, s, 19-CH_3), 0.88 (3H, d, J = 6.4, 21-CH_3), 2.01 (3H, s, OCOCH_3), 3.65 (3H, s, COOCH_3), 3.82 (1H, bs, 7-C*H*), 4.65–4.75 (1H, m, 3-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.11, 11.78, 15.39, 15.87, 18.56, 19.83, 20.23, 20.91, 21.40, 23.39, 23.58, 27.36, 28.12, 29.52, 33.56, 35.16, 35.34, 35.52, 36.38, 36.90, 39.44, 42.61, 45.69, 50.49, 51.56, 55.88, 67.78, 73.55, 170.55, 175.01.

*Methyl 3β-acetoxy-24,25-methylen-24-bishomo-5α-chol-7-en-26-oate (**22a**).* To a solution of **21a** (106 mg, 0.22 mmol) in freshly distilled pyridine (4 mL) cooled at 0 °C, dry $SOCl_2$ (0.05 mL, 0.66 mmol) was added, and the mixture was stirred at room temperature for 1 h. The mixture was poured into H_2O (15 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was sequentially washed with a saturated solution of $NaHCO_3$ (20 mL), 3 N HCl (2 × 20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness, to obtain a thick yellow oil. The crude was purified by flash chromatography eluting with petroleum ether/AcOEt (from only petroleum ether to petroleum ether/EtOAc 7 : 3) to yield **22a** as a pure white solid (66 mg, 0.14 mmol, 65%). R_f (petroleum ether/EtOAc 8 : 2): 0.23. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.51–0.52 (3H, m, 18-CH_3), 0.80 (3H, s, 19-CH_3), 0.90 (3H, d, J = 6.3 Hz, 21-CH_3), 2.02 (3H, s, OCOCH_3), 3.67 (3H, s, COOCH_3), 4.66–4.72 (1H, m, 3-C*H*), 5.14 (1H, bs, 7-C*H*). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.78, 12.88, 13.45, 17.84, 18.10, 18.86, 21.41 (2×), 22.38, 22.90, 23.71, 27.45, 27.80, 29.49, 33.77, 34.15, 35.85, 36.79, 39.40, 40.01, 43.31, 49.21, 51.47, 54.92, 55.87, 73.43, 117.27, 139.46, 170.63, 173.47.

*Methyl 3β-acetoxy-24,25-methylen-24-bishomo-5α-chol-7-en-26-oate (**22b**).* Compound **22b** was obtained following the same procedure described for **22a**, starting from the alcohol **21b** (138 mg, 0.28 mmol) in 60% yield. R_f (petroleum ether/EtOAc 8 : 2): 0.20. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.51–0.52 (3H, m, 18-CH_3), 0.80 (3H, s, 19-CH_3), 0.89 (3H, d, J = 6.4 Hz, 21-CH_3), 2.02 (3H, s, OCOCH_3), 3.65 (3H, s, COOCH_3), 4.66–4.71 (1H, m, 3-CH), 5.14 (1H, bs, 7-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.79, 12.89, 15.60, 18.74, 19.81, 20.21, 21.41 (2×), 22.87, 23.39, 27.45, 27.85, 29.57, 33.76, 34.15, 35.12, 35.68, 36.79, 39.44, 40.00, 43.32, 49.20, 51.54, 54.92, 55.89, 73.42, 117.35, 139.37, 170.62, 175.00.

*3β-Hydroxy-24,25-methylen-24-bishomo-5α-chol-7-en-26-oic acid (**23a**).* The methyl ester **22a** (39 mg, 0.08 mmol) was dissolved in 2.5% NaOH solution in MeOH (1.5 mL) and the mixture was refluxed for 5 h under magnetic stirring. The solvent was removed under vacuum, the residue was dissolved in H_2O (20 mL), acidified to pH = 3 with 3 N HCl and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with H_2O (20 mL) and brine (20 mL), dried over Na_2SO_4 and evaporated to dryness, to furnish **23a** as a white solid (35 mg, 0.08 mmol, quantitative). R_f ($CHCl_3$/MeOH 95 : 5): 0.47. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.49–0.50 (3H, m, 18-CH_3), 0.76 (3H, s, 19-CH_3), 0.87–0.89 (3H, m, 21-CH_3), 3.52–3.60 (1H, m, 3-CH), 5.12 (1H, bs, 7-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.73, 12.94, 13.81, 14.07, 17.85, 18.78, 20.95, 21.46, 22.95, 23.67, 27.80, 29.59, 31.09, 34.11, 35.66, 35.93, 37.08, 37.54, 39.47, 40.17, 43.29, 54.92, 55.86, 60.44, 70.83, 117.38, 139.46, 177.11.

*3β-Hydroxy-24,25-methylen-24-bishomo-5α-chol-7-en-26-oic acid (**23b**).* Compound **23b** was obtained following the same procedure described for **23a**, starting from the methyl ester **22b** in 90% yield (35 mg, 0.08 mmol). R_f ($CHCl_3$/MeOH 95 : 5): 0.36. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.47 (3H, s, 18-CH_3), 0.73 (3H, s, 19-CH_3), 0.84–0.86 (3H, m, 21-CH_3), 3.48–3.54 (1H, m, 3-CH), 5.10 (1H, bs, 7-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.69, 12.88, 15.65, 16.07, 18.66, 19.77, 20.11, 21.42, 22.82, 23.73, 27.81, 29.59, 30.95, 34.08, 35.07, 35.70, 37.02, 37.44, 39.45, 40.13, 43.27, 54.90, 55.85, 70.66, 117.42, 139.38, 177.75.

*3-Keto-24,25-methylen-24-bishomo-5α-chol-7-en-26-oic acid (**5–8**).* To a suspension of **23a** (40 mg, 0.09 mmol **23a**) or **23b**, (35 mg, 0.08 mg **23b**) in acetone (6 mL) at 0 °C, Jones reagent was added dropwise untill the colour of the mixture turned from green to red (4 drops). The mixture was stirred at room temperature for 2 h, then MeOH (2 mL) and H_2O (20 mL) were added. The product was extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with H_2O (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4 and evaporated under reduced pressure, providing target products **5–8**. **5–6**: 35 mg (0.08 mmol). R_f: 0.42 ($CHCl_3$/MeOH, 95 : 5). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.55–0.56 (3H, m, 18-CH_3), 0.94 (3H, d, J = 6.3, 21-CH_3), 1.01 (3H, s, 19-CH_3), 5.17 (1H, bs, 7-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.85, 12.42, 14.42, 18.74, 19.86, 21.66, 22.88, 24.42, 27.87, 29.67, 30.03, 34.36, 35.70, 35.79, 38.08, 38.73, 39.38, 42.82, 43.33, 44.20, 48.80, 54.87, 55.87, 117.04, 139.40, 179.20 (×2), 212.01. HRMS (ESI): $C_{27}H_{40}O_3$ $[M - H]^-$: calcd 412.29762, found: 412.29385. **7–8**: 26 mg (0.06 mmol). R_f: 0.41 ($CHCl_3$/MeOH, 95 : 5). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.56 (3H, s, 18-CH_3), 0.91 (3H, d, J = 6.51, 21-CH_3), 1.02 (3H, s, 19-CH_3), 5.19 (1H, bs, 7-CH). ^{13}C-NMR ($CDCl_3$, 100.6 MHz) δ: 11.85, 12.42, 16.62, 18.74, 19.86, 21.65, 22.89, 24.43, 27.87, 29.67, 30.03, 34.36, 35.11, 35.70, 35.78, 38.07, 38.73, 39.38, 42.82, 43.35, 44.23, 48.84, 54.90, 55.85, 117.00, 139.48, 180.59, 212.02. HRMS (ESI): $C_{27}H_{40}O_3$ $[M - H]^-$: calcd 412.29762, found: 412.29378. The two couples of isomers were separated by using research-type Cinchona alkaloyd-based anion exchanger type chiral stationary phase following our previous reported protocol.[20]

AlphaScreen assays

Activation of the DAF-12 receptor was determined using AlphaScreen technology. Briefly, assays were conducted in white, low volume, 384-well ProxyPlate using a final volume of 10 μL containing 10 nM glutathione transferase-tagged *ss*DAF-12-LBD protein and 30 nM biotinylated Src-1 peptide. The stimulation was carried out with different DA concentrations for 30 min at 25 °C. Luminescence was read in an EnVision 2103 microplate analyzer (PerkinElmer, USA) after incubation with the detection mix (acceptor and donor beads) for 4 h at 25 °C in the dark. Dose–response curves were performed in triplicate. To test the antagonism effect, the GST-*ss*DAF-12 and the biotinylated Rsc-1 were incubate with a fixed concentration of **1** at 0.3 μM and a dose response of compound **5** per 30 min at 25 °C.

Computational modelling

The Maestro graphical user interface and the LigPrep module (Schrödinger, LLC, New York, NY, 2020) were used to build and assign a protomeric state to the investigated compounds. The PDB code 3GYU[4] was used as source for the *ss*DAF-12 LBD structure in complex with **1**. The complex was treated using the default settings of the Protein Preparation Wizard protocol of the Schrödinger Suite. Next, the Glide SP docking program was run to obtain the best ranked putative binding poses. The grid box was centered on the co-crystallized ligand. The conformational energy of the co-crystallized pose of compound **1** and of the putative bioactive conformation of **5–8** obtained by the docking run was estimated using the Current Energy task of the Macromodel software, using the OPLS3e as force field in water solvent, while leaving all the other settings at default value.[26] In order to calculate the global minima energy of compounds **1** and **5–8** a Conformational Search analysis has been performed with the Macromodel software using the default settings, the OPLS3e force field and the water solvent. The GRID 2021 graphical user interface (version 2021.1, Molecular Discovery Ltd, UK)[21] was used to calculate the hydrophobic molecular interaction field generated by the CRY probe in the DAF-12 protein (pdb code: 3GYU). The MIF was evaluated at −1.00 kcal mol^{-1}. Afterwards, the best docking poses for compounds **1** and **5–8** previously obtained were uploaded into the GRID session.

Conflicts of interest

R.P. and A.G. are cofounders of TES Pharma. R.P. is President of TES Pharma.

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