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27-Nor-Δ^4-dafachronic acid is a synthetic ligand of *Caenorhabditis elegans* DAF-12 receptor

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ABSTRACT

27-Nor-Δ^4-dafachronic acid was prepared in nine steps and 14% overall yield by two sequential 2-carbon homologations from 20β-carboxyaldehyde-4-pregnen-3-one. Its activity was evaluated in vivo, where it rescued the Mig phenotype of *daf-9(rh50) Caenorhabditis elegans* mutants and restored their normal resistance to oxidative stress. 27-Nor-Δ^4-dafachronic acid was also able to directly bind and activate DAF-12 in a transactivation cell-based luciferase reporter assay, although it was less active than the corresponding 25*R*-and 25*S* dafachronic acids. The binding mode of the 27-Nor steroid was studied by molecular dynamics using a homology model of the *Ce*DAF-12 receptor.

The nuclear receptor DAF-12 participates in multiple essential physiological functions in nematodes, such as metabolism, homeostasis and fertility. In *Caenorhabditis elegans* (*Ce*), this receptor is also extensively associated with essential events related to animal developmental timing and life span.[1,2] Since many of the molecular and cellular pathways occurring in the nematode show analogies to corresponding pathways on higher animals,[3,4] a detailed understanding of DAF-12 function may result central to shed light on the molecular mechanisms involved in human aging and offer interesting implications for research related to human diseases.

The *Ce*DAF-12 ligands, termed dafachronic acids (DAs) are cholesterol metabolites with an acidic carboxylic group in the side chain, introduced by the cytochrome P450 oxidase DAF-9 (Fig. 1).[5] Since its discovery, several endogenous and synthetic DAs have been reported, showing that a C-3 keto group and an unsaturated double bond at C-7 or C-4 are required for an efficient *Ce*DAF-12 activation (Fig. 1). The 25*S* diastereomers of both Δ^4 and Δ^7 DAs (**1** and **2**) are more active than the corresponding 25*R* isomers, indicating that the configuration of the methyl group affects either ligand binding or complex conformation. Recently, we have investigated the binding mode of Δ^7-DAs by performing molecular dynamics simulation (MD) on a homology model of *Ce*DAF-12.[6] Besides proposing that the higher activity of the (25*S*)-Δ^7-DA isomer could be related to the strong ligand–receptor electrostatic interaction produced in this system, our model revealed that the lack of the C-25 methyl group does not impede an adequate recognition between the ligand carboxylic group and the receptor, predicting thus that 27-Nor-DAs are putative *Ce*DAF-12 ligands. As we believed that the experimental verification of this finding could expand the opportunities for design and synthesis of novel DA

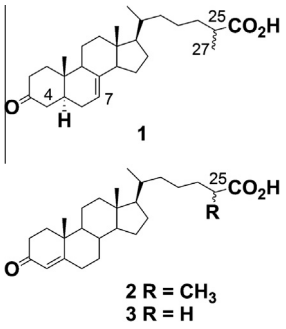

Figure 1. Structures of Δ^7- and Δ^4-dafachronic acids (**1** and **2**) and 27-Nor-Δ^4-dafachronic acid (**3**).

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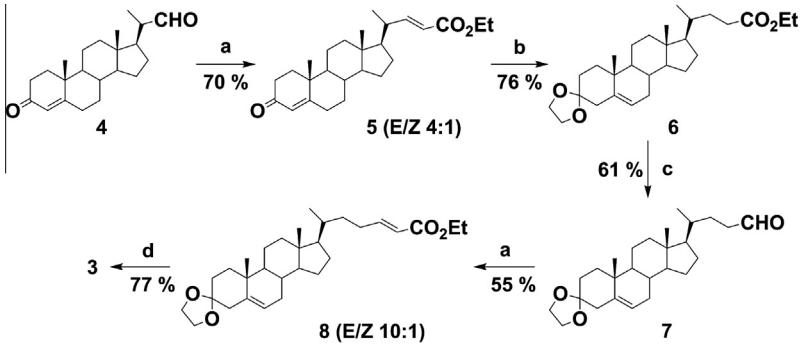

Scheme 1. Reagents and conditions: (a) $[Ph_3PCH_2CO_2Et]Br$, CH_2Cl_2–$NaHCO_3$ (aq), reflux; (b) (i) ethyl orthoformate, 1,2-ethanediol, *p*-TsOH, 25 °C; (ii) H_2, Pd-10% C, EtOAc, 1 bar, 25 °C; (c) (i) $LiAlH_4$, THF, 0 °C; (ii) PCC, $BaCO_3$, MS (3 Å), 25 °C; (d) (i) H_2, Pd 10% C, EtOAc, 1 bar, 25 °C; (ii) 5% LiOH (aq), THF–MeOH, 25 °C, (iii) *p*-TsOH, acetone, 25 °C.

analogues avoiding the requirement of diastereoselective reactions for building the side chain, we have now synthesized 27-Nor-Δ^4-DA (**3**) and evaluated its biological activity both in vivo and in vitro.

27-Nor-Δ^4-DA (**3**) was prepared as outlined in Scheme 1. The strategy employed was based on two 2-carbon homologations of the commercially available aldehyde **4**, the key steps for this transformation being a Wittig reaction followed by regioselective reduction of the newly generated double bond. The Wittig reaction was carried out with carbethoxymethylenetriphenylphosphonium bromide and aqueous $NaHCO_3$ in dichloromethane, to give **5** as an *E*/*Z* mixture (4:1, determined by ^{1}H NMR). Treatment of **5** with 1,2-ethanediol gave the corresponding ethylene ketal derivatives, and the mixture was hydrogenated under mild conditions(H_2, Pd 10% C, 1 bar) to give compound **6**. Reduction of the C-24 ester group with $LiAlH_4$, followed by oxidation with pyridinium chlorochromate in the presence of barium carbonate gave aldehyde **7**. A second Wittig reaction in the same conditions as above, gave the unsaturated ester **8** (*E*/*Z* > 10:1 by ^{1}H NMR). Regioselective hydrogenation gave the 27-Norcholestenoic ester that was submitted to basic hydrolysis (LiOH in THF–methanol–water) and ethylene ketal removal (*p*-TsOH in acetone) to give **3** in 14% overall yield from **4** (see Supplementary data, Materials and methods).

C. elegans daf-9(rh50) mutants have penetrant heterochronic delays in L3 gonadal leader cell migrations (Mig phenotype), reduced fecundity and are slightly short-lived presumably because DAF-12 is not fully activated.[7,8] Thus, to evaluate the in vivo activity of 27-Nor-Δ^4-DA (**3**) we initially evaluated the rescue of the Mig phenotype of *daf-9(rh50)* mutants. The *daf-9(rh50)* and N2 wild type worms were grown in the presence of 250 nM 27-Nor-Δ^4-DA at 23 °C,[8–10] or ethanol as control, and the percentage of worms with wild type gonads was scored after 3 days. The results showed that in *daf-9(rh50)* mutants, the Mig phenotype was extensively rescued by 27-Nor-Δ^4-DA (Fig. 2A). Moreover, we also found that treated *daf-9* mutants were indistinguishable from wild type adults: a 100% bypassed dauer, exhibited normal gonadal migration and were able to produce progeny.

It has been previously shown that *daf-9* adults exhibit significantly stronger resistance to oxidative stress compared to wild type worms.[8] Then, to further analyze the 27-Nor-Δ^4-DA activity in vivo we evaluated the resistance to oxidative stress of *daf-9(rh50)* mutants in the presence or absence of this steroid. Thus, *daf-9(rh50)* and N2 wild type worms were kept at 23 °C for an hour in plates containing or not 27-Nor-Δ^4-DA and then H_2O_2 was added in millimolar level. The survival was scored at 2 h intervals during an 8 h period, showing that *daf-9(rh50)* were substantially more resistant compared with wild type animals (Fig. 2B). Defective *daf-9* mutants supplemented with 27-Nor-Δ^4-DA behaved like wild type animals against the oxidative stress. Thus, the treatment with 27-Nor-Δ^4-DA at 250 nM restored normal resistance of *daf-9(rh50)*. While *ca.* 80% of mutants were alive upon 6 h of

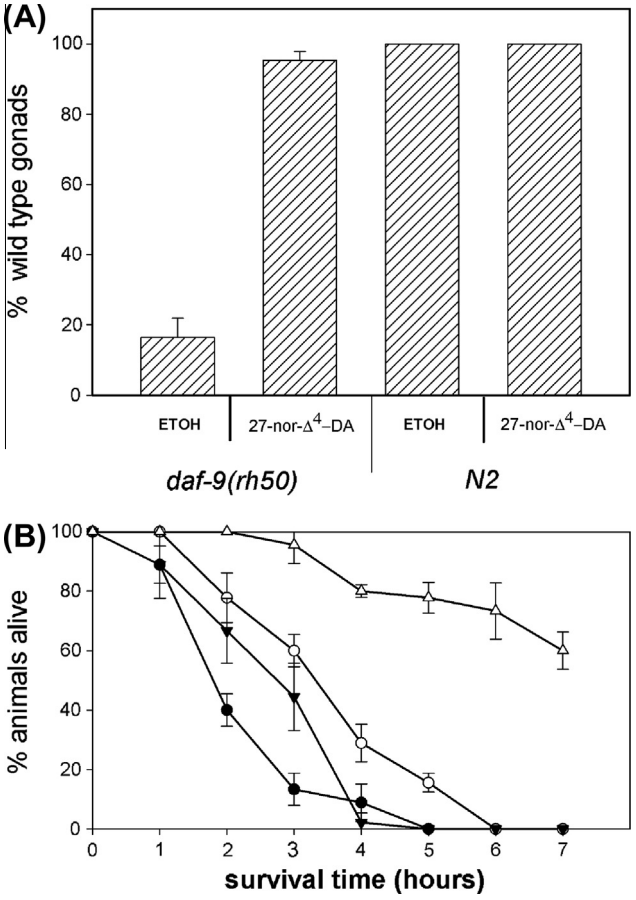

Figure 2. (A) 27-Nor-Δ^4-DA (**3**) rescued the Mig phenotype of *daf-9(rh50)* mutants. *daf-9(rh50)* and N2 wild type worms developed in presence of 250 nM 27-Nor-Δ^4-DA,[9] or ethanol as control, at 23 °C and the percentage of worms with reflexed gonadal arms were scored after 3 days. Mean and standard deviations of three independent experiments with $n > 60$ worms are shown; (B) oxidative stress resistance of *daf-9* mutants depends on the presence of 27-Nor-Δ^4-DA (**3**). *daf-9(rh50)* and N2 young adults were transferred to plates containing 15 mM H_2O_2 and incubated at 23 °C in the presence of 250 nM 27-Nor-Δ^4-DA (filled triangles and circles, respectively), or ethanol as control (unfilled triangles and circles, respectively). Mean and standard deviations of three independent experiments with $n > 60$ worms are shown.

H_2O_2 treatment, they did not survive in the presence of this steroid. Moreover, 27-Nor-Δ^4-DA even decreased the percentage of survival N2 respect to untreated N2 animals. In summary, the in vivo results reveal that 27-Nor-Δ^4-DA has the ability of behaving as DA, reversing the associated effects produced by the deficient biosynthesis in *daf-9* mutants.

Next, to investigate whether 27-Nor-Δ^4-DA is able to directly bind and activate DAF-12, we used a transactivation cell-based luciferase reporter assay. HEK 293T cells were co-transfected with the pCMX-Gal4-DAF-12LBD expression vector, which expresses a fusion protein of the Gal4 DNA-binding domain with the LBD of the nuclear hormone receptor DAF-12,[5] and the pGL2 luciferase reporter construct containing the GAL4 upstream activating sequence (UAS). Transfected cells were incubated with increasing concentrations of 27-Nor-Δ^4-DA. Results from Figure 3 show a dose-dependent increase of luciferase expression in response to the DA analogue **3**, with an approximate EC_{50} value in the order of 10 μM.[11] These results confirm that 27-Nor-Δ^4-DA actually mimcs a *bona fide* DAF-12 ligand. However, its potency is lower than that observed for C-25 methyl Δ^4-DAs ((25*S*)-Δ^4-DA: EC_{50} = 0.1 μM and (25*R*)-Δ^4-DA:$EC_{50} \geqslant 1$ μM),[5] concluding that the lack of the C-25 methyl decreased ligand affinity, resulting in a less potent steroid. It is noteworthy that this result qualitatively correlates with our previous in silico simulations, in which we used the thermodynamic integration method to calculate the relative binding free energy for three Δ^7-DAs.[6] Those results showed that the 27-Nor-Δ^7-steroid had a smaller relative binding free energy compared to the 25*R* and 25*S* Δ^7-DAs.

The ligand binding mode of 27-Nor-Δ^4-DA (**3**) was investigated carrying out a 20 ns MD simulation of the *Ce*DAF-12/27-Nor-Δ^4-DA complex. The initial complex structure was obtained from the coordinates of the *Ce*DAF-12/(25*R*)-Δ^7-DA model,[6] superimposing carbon atoms of the steroid skeleton and maintaining the side chain in the fully extended conformation. The visual inspection of the trajectory and the RMSD of ligand atoms relative to the initial structure, revealed that no major changes in the position occurred during the simulation. As was expected, while the non-polar steroid skeleton is surrounded by hydrophobic residues, both polar groups established strong interactions with the polar residues of the DAF-12 binding pocket, allowing an adequate ligand-receptor interaction (Fig. 4). On one side, the 3-keto group forms a stable hydrogen bond with the Gln638 during all the simulated time, with average values very similar to those observed in Δ^7-DAs systems. At the other end of the steroid molecule, the terminal carboxyl group participates in an extensive electrostatic network involving three arginines (Arg564, Arg598 and Arg564) and one asparagine (Asn532). As in the case of the Δ^7 steroids, simultaneous interactions were observed between the carboxylic group and the three guanidinium groups, which maintain the steroid firmly clamped within the ligand binding pocket. However, at variance with the 27-Nor-Δ^7-DA system, where three different conformations of the steroid side chain are explored along the trajectory, the steroid side chain of 27-Nor-Δ^4-DA rapidly acquired a torsioned conformation that remained stable until the end of the simulation. Thus, although in both 27-Nor-Δ^4-DA and 27-Nor-Δ^7-DA the lack of C-25 methyl moiety does not impede the strong interaction with the arginines residues, the MD simulation reveals that the conformation and dynamic behavior of the side chain markedly depends on the position of the double bond in the steroid nucleus, which affects its overall shape. Differences in the conformation of the steroid side chain have been observed in the crystal structures of the *Strongyloides stercolaris* DAF-12 complexed with (25*R*)-Δ^7-DA (fully extended conformation) and with (25*R*)-Δ^4-DA (torsioned conformation),[12] thus supporting the conclusions from our homology model.

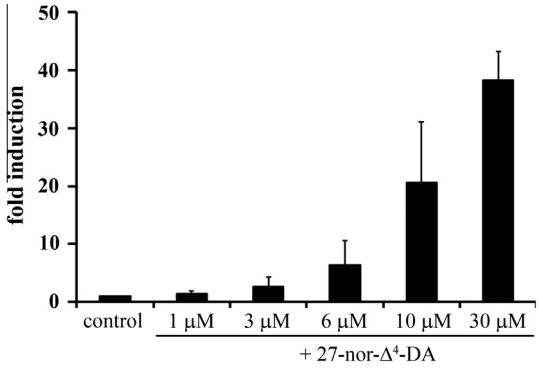

Figure 3. 27-Nor-Δ^4-DA (**3**) mimics a *bona fide* DAF-12 ligand. HEK293 cells were co-transfected with the pCMX-Gal4-DAF-12LBD, the pGL2 luciferase reporter construct and the pCMV-LacZ as control of transfection. Then, cells were incubated for 20 h with increasing concentrations of 27-Nor-Δ^4-DA.[11] Luciferase activity was measured and normalized against β-galactosidase activity. Values are expressed as fold induction relative to the control. Mean and standard deviations of three independent experiments are shown.

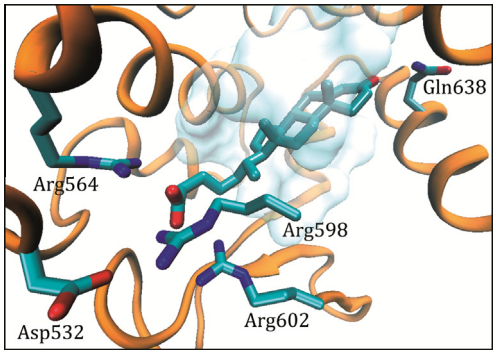

Figure 4. Ligand binding mode of 27-Nor-Δ^4-DA. Representative snapshot of DAF-12/27-Nor-Δ^4-DA complex during the molecular dynamics simulation showing the polar interactions between the steroid C-3 keto group and Gln638 and between the steroid C-26 carboxyl group and Arg564, Arg598 and Arg602 arginines. Non-polar residues surrounding the steroid carbon skeleton are shown as cyan shadows.

Finding that 27-Nor-Δ^4-DA behaves as the natural dafachronic acids both in vivo and in vitro, increases the number of potential structures with the ability to modulate the DAF-12 receptor, as steroids with a linear (unbranched) side chain are more accessible from a synthetic standpoint, than those with a C-25 stereocenter. This was initially predicted in silico, by MD simulations on a molecular model of the ligand binding domain of *Ce*DAF-12 and has now been confirmed experimentally. The DAF-12 receptor belongs to the nuclear receptor family and shares similar structure with the human homologs LXRα and LXRβ, both of which are involved in lipid regulation and are targets of different cholesterol metabolites. In this context, 27-Nor-Δ^4-DA appears as an interesting lead to analyze the ability of 27-Nor-steroids as putative LXR modulators.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.071.

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