Synthesis and Activity of Dafachronic Acid Ligands for the *C. elegans* DAF-12 Nuclear Hormone Receptor

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The nuclear hormone receptor DAF-12 from *Caenorhabditis elegans* is activated by dafachronic acids, which derive from sterols upon oxidation by DAF-9, a cytochrome P450. DAF-12 activation is a critical checkpoint in *C. elegans* for acquisition of reproductive competence and for entry into adulthood rather than dauer diapause. Previous studies implicated the (25*S*)-Δ^7-dafachronic acid isomer as the most potent compound, but the (25*S*)-Δ^4-isomer was also identified as an activator of DAF-12. To explore the tolerance of DAF-12 for structural variations in the ligand and to enable further studies requiring large amounts of ligands for DAF-12 and homologs in other nematodes, we synthesized (25*R*)- and (25*S*)-isomers of five dafachronic acids differing in A/B-ring configurations. Both the (25*S*)- and (25*R*)-Δ^7-dafachronic acids are potent transcriptional activators in a Gal4-transactivation assay using HEK-293 cells, with EC_{50} values of 23 and 33 nM, respectively, as are (25*S*)- and (25*R*)-Δ^4-dafachronic acids, with EC_{50} values of 23 and 66 nM, respectively. The (25*S*)- and (25*R*)-Δ^5-isomers were much less potent, with EC_{50} values approaching 1000 nM, and saturated 5α- and 5β-dafachronic acids showed mostly intermediate potencies. Rescue assays using *daf-9*-null mutants confirmed the results from transactivation experiments, but this *in vivo* assay accentuated the greater potencies of the (25*S*)-epimers, particularly for the (25*S*)-Δ^7-isomer. We conclude that DAF-12 accommodates a large range of structural variation in ligand geometry, but (25*S*)-Δ^7-dafachronic acid is the most potent and probably biologically relevant isomer. Potency derives more from the A/B-ring configuration than from the stereochemistry at C-25. **(*Molecular Endocrinology* 23: 640–648, 2009)**

The life cycle of *Caenorhabditis elegans* allows for arrested development in a protected dormant or dauer diapause during periods of stress or starvation. When favorable conditions return, a pathway is activated to complete the second larval molt and to restore reproductive competence. The genes responsible for the entry and exit from the dauer diapause, termed the *daf* genes (for dauer formation), have been the topic of intense study, and the functions of many proteins encoded by these genes have been identified (1). In particular, two of the terminal genes in this pathway, *daf-9* and *daf-12*, encode a cytochrome P450 enzyme and a nuclear hormone receptor, respectively (2–4).

Given that *C. elegans* is auxotrophic for cholesterol (2), we reasoned that the environmental nutrient necessary for the cascade of events culminating in exit from the dauer diapause was a sterol, which was oxygenated by DAF-9 to form the ligand for DAF-12. Using this model, which intriguingly parallels the oxygenation of cholesterol to androgens and estrogens during reproductive maturation in mammals, we previously identified two compounds extracted from worms, which we have called the Δ^7- and Δ^4-dafachronic acids (7- and 4-cholesten-3-one-26-oic acids, *I* and *II*, Fig. 1). Our prior studies suggested that (25*S*)-Δ^7-dafachronic acid was the most potent and important endogenous ligand with nanomolar affinity; however, the dafachronic acids were previously unknown compounds (3). We synthesized authentic Δ^4-dafachronic acid and confirmed its potency, but we did not have access to synthetic Δ^7-dafachronic

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Abbreviations: DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; LBD, ligand-binding domain; MPLC, medium-pressure liquid chromatography; NMR, nuclear magnetic resonance; RT, room temperature; THF, tetrahydrofuran.

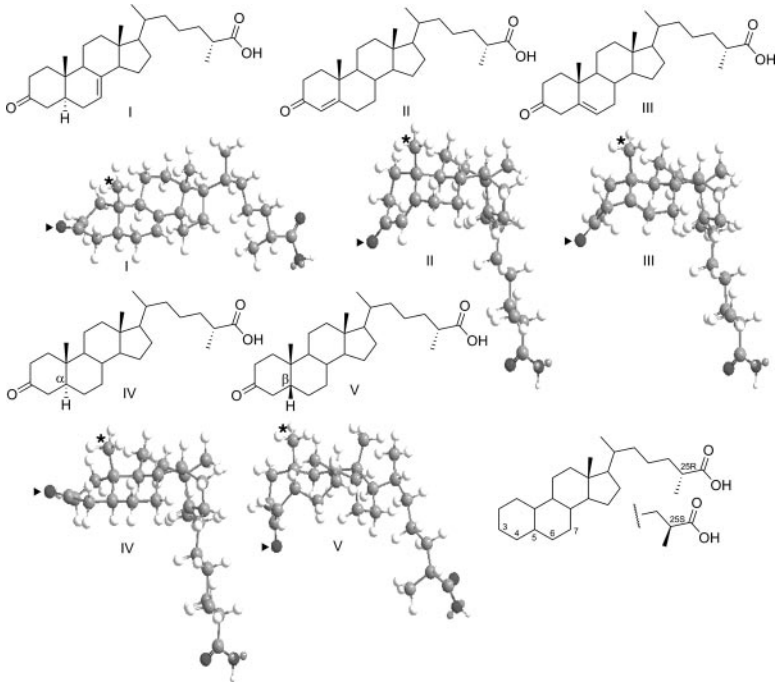

FIG. 1. Structures of dafachronic acids synthesized in this study. The 25*R* isomers of each compound are shown in *I-V*, with important carbon atoms numbered and stereochemical features defined at C-5 and C-25. *Beneath* the structures are ball-and-stick models, which illustrate the differences in A/B-ring geometries among these compounds. The 19-methyl groups are highlighted by *asterisks*, and the 3-ketone oxygen atoms are indicated by *arrowheads*. Models were rendered by ChemBio 3D Ultra 11.0 after energy minimization with MM2.

acid, and we did not test other sterol acids with additional configurations of the A/B ring system.

Consequently, we have developed a versatile synthetic scheme suitable for large-scale preparation of multiple dafachronic acids from readily available and inexpensive diosgenin (*VI*). After synthesizing compounds *I–V*, we explored how the position of a double bond in the A/B ring system and the stereochemistry at C-5 and C-25 influence the potency of dafachronic acid ligands for DAF-12 using transactivation and dauer rescue assays.

Results

Synthesis of dafachronic acids

The strategy employed to access a repertoire of dafachronic acids centered on the common intermediate *X*, which derives from diosgenin (*VI*) in four steps (Scheme 1 in Fig. 2). Conversion of *X* to (25*R*)-Δ^5- and Δ^4-dafachronic acids (*III* and *II*) require only a few additional steps (Scheme 2 in Fig. 3). The main hurdle in preparation of (25*R*)-*III* and *II* is to oxidize the 3-hydroxyl without isomerization of the double bond using 2-iodoxybenzoic acid to access *III* or with clean isomerization to the conjugated enone in *II* using Oppenauer oxidation. The terminal steps in conversion of intermediate 3-keto-26-alcohols to the corresponding 3-keto-26-acids differs for the two compounds to prevent isomerization of the double bond en route to (25*R*)-*III*.

Several routes were attempted to introduce the 5α-reduced-Δ^7-system found in (25*R*)-*I*. Hydroboration and oxidation of *X* gave the 5α-reduced-6-ketosterol, which could be functionalized to the Δ^7-enone via bromination and elimination but not using phenylselenyl chloride/hydrogen peroxide. Deoxygenation of this enone, however, could not be achieved. Allylic bromination of *X* with 1,3-dibromo-5, 5-dimethylhydantoin, elimination to the 5,7-diene, and selective 5α-hydrogenation of the Δ^5-olefin afforded the 5α-reduced Δ^7-system found in (25*R*)-*I*, but the bromination gave low yields and could not be scaled to more than 15 mg *V*. In contrast, allylic oxidation, hydrogenation, reduction, and elimination (4) installed the 5α- reduced Δ^7-system in good yield (Scheme 3 in Fig. 4).

These routes to dafachronic acids retain the (25*R*)-stereochemistry found in diosgenin, necessitating a procedure for inverting the stereochemistry at C-25. Routes via the Δ^{25}-olefins using chiral boranes to selectively hydroborate the terminal olefin were envisioned; however, elimination of the 26-alcohols proceeded in low yield with side products under several conditions. The 26-alcohols were then oxidized to the 26-aldehydes and isomerized using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to 1:1 mixtures of the (25*R*)- and (25*S*)-aldehydes by

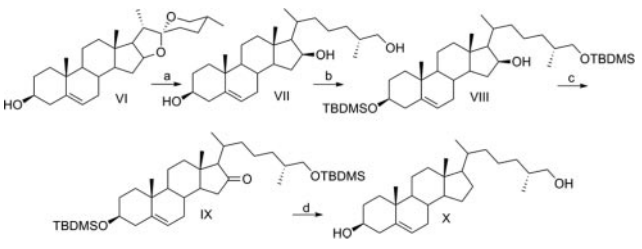

FIG. 2. Scheme 1: a, Zn(Hg), HCl, ethanol; b, tert-butyl-dimethylsilyl chloride (TBDMSCl), DBU, THF; c, Dess-Martin periodinane, CH_2Cl_2; d, $NH_2NH_2 \cdot HCl$, $NH_2NH_2 \cdot H_2O$ diethylene glycol.

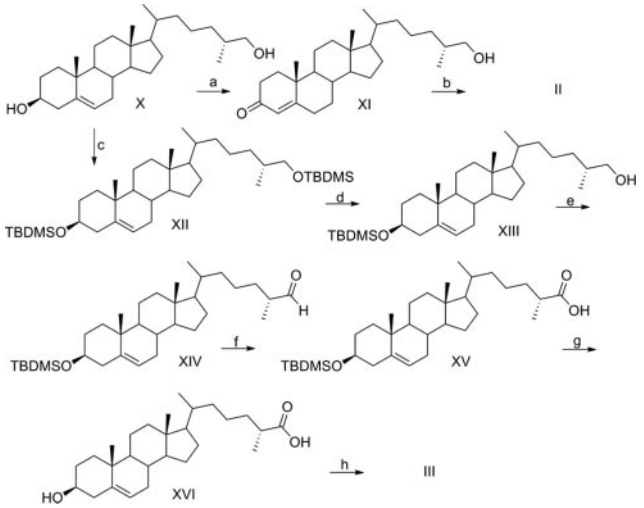

FIG. 3. Scheme 2: a, Al(O*i*-Pr)$_3$, 1-methyl-4-piperidone, toluene; b, Jones reagent, 0 C; c, tert-butyl-dimethylsilyl chloride (TBDMSCl), DBU, THF, 24 h; d, CSA, MeOH, 0 C; e, Dess-Martin periodinane, CH_2Cl_2; f, $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene; g, TBAF, THF; h, iodoxybenzoic acid (IBX), dimethylsulfoxide/THF (1:1).

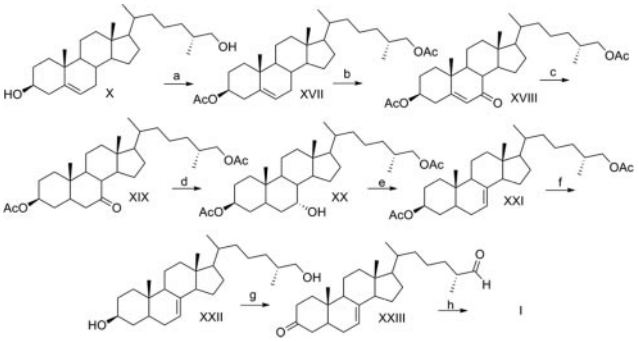

FIG. 4. Scheme 3: a, Ac_2O, pyridine, dimethylaminopyridine (DMAP); b, 3,5 dimethylpyrazole, CrO_3, CH_2Cl_2, −15 C; c, Pd/C, H_2, EtOAc; d, L-selectride, THF, −78 C; e, Burgess reagent, benezene, reflux; f, KOH, MeOH; g, PCC, NaOAc; h, $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene.

nuclear magnetic resonance (NMR) (Fig. 5), which were converted to the 26-alcohols or acids by further reduction or oxidation, respectively (Scheme 4 in Fig. 6). These mixtures of diastereomers could not be separated using silica gel chromatography or reverse-phase HPLC, and attempts to form esters with chiral alcohols or acids to improve separations gave poor yields. Instead, the diastereomeric 26-alcohols were kinetically resolved by selective acetylation of the (25*S*)-isomers with vinyl acetate catalyzed by *Pseudomonas cepacia* lipase in chloroform. The (25*S*)-acetates were easily separated from the unreacted (25*R*)-alcohols and carried forward to the (25*S*)-dafachronic acids (see supplemental data and schemes, especially Schemes 4a and 4b, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

Finally, generation of the (25*R*)- and (25*S*)-5α-reduced dafachronic acids *IV* was achieved by catalytic hydrogenation of any Δ^4-intermediates or *II* with $Pd/CaCO_3$. The corresponding 5β-reduced dafachronic acids *V* were prepared from *II* using ammonium formate reduction catalyzed by Pd on carbon black (Scheme 5 in Fig. 7).

Gal4-transactivation assays

In our previous study, we identified two dafachronic acids characterized by having 3-keto-Δ^4- or Δ^7-configurations, but

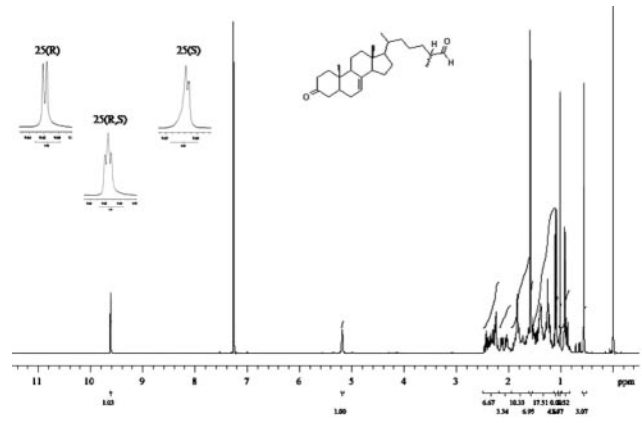

FIG. 5. Epimerization and resolution of dafachronic acid intermediates. The ^{1}H-NMR spectrum of aldehydes *XXIII* before and after epimerization and resolution. *Inset* shows expansion of aldehyde (C*H*O) proton resonance, which is a clean doublet as predicted for a single 25(*R*)-isomer and becomes overlapping doublets (pseudotriplet) after isomerization. The 25(*S*)-isomer, obtained after enzymatic kinetic resolution of epimeric alcohols and oxidation, also shows a doublet.

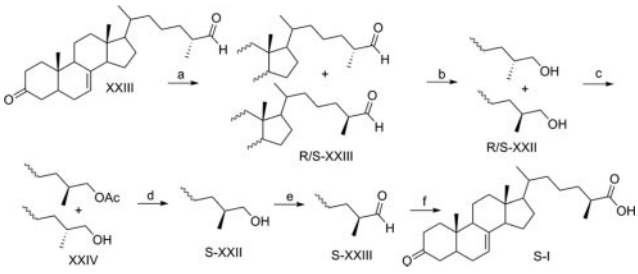

FIG. 6. Scheme 4: a, DBU, THF, 24 h; b, $NaBH_4$, methanol, 0 C; c, *P. cepacia* lipase, vinyl acetate, $CHCl_3$; d, KOH, MeOH, reflux; e, PCC, NaOAc; f, $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene.

authentic standards of neither Δ^7-dafachronic acids nor structurally similar compounds were available. In addition, 5-cholesten-3β-ol-26-oic acid also activated DAF-12 and rescued the dauer phenotype (3, 5), suggesting that DAF-12 could be activated by compounds with other A/B ring configurations. We first employed the convenient Gal4-transactivation assay to test the activation of DAF-12 by the panel of dafachronic acids *I-V* in HEK-293 cells to characterize the structural features required for DAF-12 transactivation.

All dafachronic acids transactivated the nuclear receptor DAF-12 but with a wide range of potencies (Table 1). As proposed previously, Δ^7-dafachronic acids *I* are the most potent activators of DAF-12 in this assay, with EC_{50} values of 23 and 33 nM for the (25*S*)- and (25*R*)-isomers, respectively. The (25*S*)-Δ^4-dafachronic acid *II* is equipotent to Δ^7-acids *I*, with an EC_{50} of 23 nM, but the (25*R*)-isomer of *II* is significantly less potent, with an EC_{50} of 66 nM. In contrast, the Δ^5-dafachronic acids *III* are significantly less potent than acids *I* and *II*, with EC_{50} values approaching 1000 nM. These data quantitatively confirm our proposal that the most potent endogenous activators of DAF-12 are the Δ^7- and Δ^4-dafachronic acids *I* and *II*, with the (25*S*)-isomers significantly more potent than the (25*R*)-isomers only for *II*. Although DAF-12 is activated by compounds *III*, metabolism of these 3-hydroxy-Δ^5-sterols to more potent ligands *in vivo* might account for much of their biological activity.

To determine whether the superior potency of the Δ^7-dafachronic acids compared with the Δ^4-isomers derives from the 5α-reduced *trans*-ring fusion or the location of the double bond in

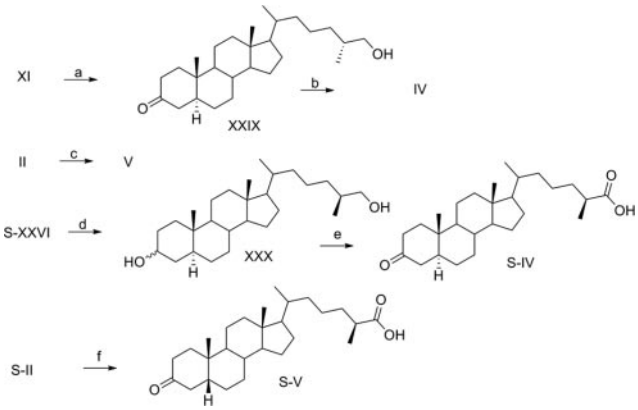

FIG. 7. Scheme 5: a, $PD/CaCO_3$, isopropanol, H_2; b, Jones reagent, 0 C; c, Pd/C, ammonium formate, MeOH, reflux; d, $Pd/CaCO_3$, acetonitrile:isopropanol (2:1), H_2; e, same as b; f, same as c.

TABLE 1. Transactivation of Gal4-DAF-12-LBD by dafachronic acid isomers in HEK-293 cells

Isomer	EC_{50} (nM)	Replicates	*P* value [25(*S*) *vs.* 25(*R*)]
Δ^7- (*I*)			0.2
(25*S*)	23 ± 11	3	
(25*R*)	33 ± 6	3	
Δ^4- (*II*)			0.00001
(25*S*)	23 ± 6	6	
(25*R*)	66 ± 12	6	
Δ^5- (*III*)			0.0002
(25*S*)	940 ± 75	5	
(25*R*)	524 ± 124	5	
5α- (*IV*)			0.005
(25*S*)	474 ± 119	4	
(25*R*)	192 ± 50	4	
5β- (*V*)			0.001
(25*S*)	329 ± 183	5	
(25*R*)	1847 ± 647	5	

Data represent means ± SD.

the A/B-ring system, we prepared the saturated 5α- and 5β-reduced dafachronic acids *IV* and *V* and tested these compounds as DAF-12 activators. Both the 5α- and 5β-reduced acids showed potencies mostly intermediate to the Δ^4- and Δ^5-isomers in the Gal4-transactivation assay, with EC_{50} values of 200-1800 nM (Table 1). The 5β-dafachronic acids were the only compounds for which the (25*S*)-isomer was convincingly more potent than the (25*R*)-isomer in this assay. These data demonstrate that both the 5α-reduced *trans*-ring fusion and the Δ^7-olefin both contribute to the potency of dafachronic acids, more than the stereochemistry at C-25.

Dauer rescue assays

Because Gal4-transactivation assays employ mammalian cells using a convenient, but artificial Gal4-DBD/DAF-12-ligand-binding domain (LBD) fusion construct, it is possible that results from these chimeric receptors may not precisely reflect the potencies of ligands with endogenous DAF-12 in *C. elegans*. Therefore, we performed dauer rescue assays to assess the potencies of these ligands *in vivo*. In this experiment, we supplemented the *daf-9*-null, constitutive dauer mutants with dafachronic acids and scored the percentage of worms rescued from dauer diapause. Similar to the results obtained from the Gal4-transactivation assay, the Δ^7- and Δ^4-dafachronic acids *I* and *II* showed significantly higher potency than the Δ^5-acids *III* (Table 2). In this assay, however, the (25*S*)-isomers were substantially more potent than the (25*R*)-isomers, and the Δ^7-dafachronic acids were convincingly more potent than the Δ^4-isomers. The 5α- and 5β-reduced dafachronic acids were likewise intermediate in potency between the Δ^5-isomers and the Δ^7-isomers, with the (25*R*)-5β-reduced compound being the least potent. These data, obtained with a more physiological assay, confirm that the 5α-reduced *trans*-ring fusion, the Δ^7-configuration in the A/B ring, and the (25*S*)-stereochemistry all contribute to the potency of DAF-12 ligands.

TABLE 2. Rescue of daf-9 (dh6) dauer phenotype by dafachronic acid isomers

Treatment	Dose (nM)	Rescue (%)	n
Vehicle		0	287
Δ^7-Dafachronic acid (*I*)			
(25*S*)	10	16	43
	50	98	158
	100	100	210
	500	100	57
	1000	100	72
(25*R*)	10	0	95
	50	0	87
	100	1	138
	500	99	204
	1000	100	199
Δ^4-Dafachronic acid (*II*)			
(25*S*)	10	0	38
	50	0	46
	100	17	42
	500	100	182
	1000	100	141
(25*R*)	500	16	110
	1000	50	84
	10000	100	113
Δ^5-Dafachronic acid (*III*)			
(25*S*)	1000	0	146
	2500	95	88
	5000	94	96
	10000	100	129
(25*R*)	1000	0	137
	2500	97	145
	5000	93	98
	10000	100	111
5α-Dafachronic acid (*IV*)			
(25*S*)	10	0	48
	50	0	77
	100	0	89
	500	19	78
	1000	97	202
	5000	98	174
	10000	98	104
(25*R*)	10	0	81
	50	0	79
	100	1	113
	500	29	121
	1000	99	181
	5000	96	130
	10000	97	149
5β-Dafachronic acid (*V*)			
(25*S*)	10	0	92
	50	0	58
	100	0	82
	500	18	66
	1000	97	172
	5000	99	139
	10000	97	138
(25*R*)	1000	0	76
	5000	6	87
	10000	79	66

Discussion

Previously, we showed that in the presence of DAF-9, the 3-keto-Δ^7-sterol lathosterone was more potent than either la-

thosterol or 4-cholesten-3-one in DAF-12 transactivation assays and in rescue assays with *daf-9*-null, constitutive dauer mutants. Compounds with mass spectra consistent with monounsaturated dafachronic acids were isolated from worms, and microsomes containing DAF-9 metabolized 4-cholesten-3-one primarily to the (25*S*)-26-alcohol and then to the 26-acid (3). These data suggested but did not prove that (25*S*)-Δ^7-dafachronic acid was the major endogenous ligand for the *C. elegans* DAF-12 nuclear hormone receptor. The structure-activity studies presented here support this conclusion, at least considering the series of structurally similar compounds analyzed. We obtained equivalent results with another sample of (25*S*)-Δ^7-dafachronic acid (kindly provided by Dr. Elias J. Corey) (4), which was prepared in a different laboratory by a different route.

In addition to the Δ^7-isomers, we also show that DAF-12 accommodates a variety of compounds similar to dafachronic acid ligands, an observation that parallels the relaxed substrate specificity of the cytochrome P450 DAF-9 (3) (Cummins, C. L., D. L. Motola, and D. J. Mangelsdorf, unpublished data). This flexibility in ligand processing and binding, in turn, might enable the animal to use a variety of sterols as raw materials to signal the return of favorable conditions and to initiate exit from the dauer diapause.

The potencies of the structurally similar ligands studied herein, however, vary over two orders of magnitude. In most but not all cases, the (25*S*)-isomers are more potent than the (25*R*)-isomers, but the magnitude of this difference varies with the A/B-ring configuration. For compounds *I–IV*, the ring fusion forces the molecule into the flat geometry of a twist-boat conformation or a *trans*-decalin. For compound *V*, the 5β-reduction forces the A-ring nearly perpendicular to the B-ring as found in bile acids, yet DAF-12 is still activated despite this large structural change. Nevertheless, *III* and *V* are the least potent compounds in this study, particularly the (25*R*)-isomers. Although DAF-12 tolerates significant structural variation in the A/B-ring geometry, the 5α-reduced stereochemistry and *trans*-decalin ring system, particularly the (25*S*)-isomer with the Δ^7-olefin, optimizes potency.

The qualitative agreement between the Gal4-transactivation and dauer rescue assays is reassuring, and the quantitative differences do not detract from our conclusions. Reasons for these discrepancies might include differences in permeability and metabolism of these ligands in *C. elegans* compared with HEK-293 cells. In addition, only the DAF-12 LBD is used in the Gal-4 transactivation assay, precluding activation via the amino-terminal (AF-1) domain in these experiments. Finally, the endogenous coactivators and transcriptional machinery in *C. elegans* and HEK-293 cells are different, so we would not anticipate exactly the same results in both assay systems.

Homologs of DAF-12 appear to be present in several nematode species, including hookworms and roundworms that infect human beings, livestock, and crops. Access to a repertoire of dafachronic acids, therefore, might translate to important medicinal and agricultural uses, particularly in developing countries. Our versatile synthetic scheme allows for production of many different dafachronic acids from inexpensive starting materials using methods that are suitable for expansion to large-scale production; we have prepared (25*R*/*S*)-*I* on a gram scale. Our data also indicate that, at least for the most potent Δ^4- and Δ^7-isomers, the more accessible (25*R*)-epimers are sufficiently potent to be used experimentally and pharmacologically. Additional synthetic approaches to specific dafachronic acids are emerging (4, 6–8), indicating interest in these compounds and suggesting that our methods could be optimized further. Consequently, this signaling pathway and the physiology elucidated in the study of a model organism may have important implications in agriculture and medicine.

Materials and Methods

General methods and reagents

The firefly luciferase substrate luciferin is from Molecular Probes (Eugene, OR); the β-galactosidase substrate *O*-nitrophenyl-β-D-galactopyranoside is from Diagnostic Chemicals Ltd. (Oxford, CT). Diosgenin was obtained from Steraloids (Newport, RI), and most chemicals and other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and Pierce (Rockford, IL).

Gal4 transactivation assay

Gal4 transactivation of DAF-12 was performed as described (3). Briefly, HEK-293 cells were transfected in 96-well plates with plasmids as follows: 50 ng MH100x4-tk-luc reporter, 10 ng CMX-β-galactosidase to control for transfection efficiency, and 15 ng CMX-Gal4-DAF-12LBD, which expresses a fusion protein of the Gal4 DNA-binding domain with the LBD of the nuclear hormone receptor DAF-12 (referred to as Gal4-DAF-12-LBD). The ligands were added 6 h after calcium phosphate transfection, and luciferase and β-galactosidase activities were measured 16–24 h after the addition of dafachronic acids or vehicle control.

Dauer rescue assay

Dauer rescue assay was performed as described (3). Vehicle control or dafachronic acid stock solutions were diluted with 0.1 ml 5×OP50 bacteria from an overnight culture to achieve the indicated concentration of ligands, and the mixtures were loaded on agar plates prepared with 4 ml nematode growth medium. After the bacteria pads were formed, about 15 daf-9 (dh6, dhEx24) gravid adults were introduced to the plates and allowed to lay eggs for 3–6 h, after which the adults were removed. The plates were stored at 23 C for 48 h, and the green fluorescent protein-positive progeny were identified under fluorescence dissection microscope. Rescue of daf-9 (dh6) dauer phenotype was scored after 12–24 h and represented as the percentage of gravid adults from all progeny.

Synthesis of dafachronic acids from diosgenin

Scheme 1

(25R)-Cholest-5-ene-3β,16β,26-triol (VII). To a refluxing solution of diosgenin (*VI*) (4.0 g, 9.6 mmol) and zinc amalgam (120 g, freshly prepared from 2 g $HgCl_2$ and 120 g mossy zinc (Aldrich) in 300 ml water with 400 ml 95% ethanol) was added concentrated HCl (120 ml) over 4 h at room temperature (RT), and heating was then continued for 30 min. The reaction mixture was decanted from zinc, cooled to RT, diluted with water, and thoroughly extracted with $CHCl_3$. The combined extracts were washed with water, dried over Na_2SO_4, and evaporated to white solid. The solid was subjected to medium-pressure liquid chromatography (MPLC) on silica gel with 3:2 ethyl acetate-hexanes. Evaporation of solvent gave *VII* (9) (2.94 g; 74%).

(25R)-3β,26-Bis(tert-butyldimethylsilyloxy)cholest-5-en-16β-ol (VIII). To a solution of *VII* (2.92 g, 7.0 mmol) in 40 ml tetrahydrofuran (THF)

was added *tert*-butyl dimethylsilylchloride (4.2 g, 27.9 mmol) under nitrogen. The mixture was stirred for 15 min at RT, and DBU (4.03 g, 26.5 mmol) was slowly added, and the resulting mixture was stirred at RT for 16 h. The reaction mixture was diluted with 100 ml water and extracted with ethyl acetate. The combined extracts were washed with water, dried over Na_2SO_4, and evaporated to dryness. The residue was subjected to MPLC on silica gel with 5% ethyl acetate in hexanes. Evaporation of solvent afforded *VIII* (10) (4.2 g, 95%).

(25R)-3β,26-Bis(tert-butyldimethylsilyoxy)cholest-5-en-16-one (IX). A solution of *VIII* (3.31 g, 5.1 mmol) in methylene chloride (50 ml) was treated with Dess Martin periodinane (2.38 g, 5.6 mmol). The reaction was stirred at RT for 6 h, quenched with saturated $Na_2S_2O_3$ solution, diluted with water, and extracted with ethyl acetate. The organic layers were dried over Na_2SO_4, filtered, and evaporated to dryness. The crude product was subjected to MPLC on silica gel with 4% ethyl acetate in hexanes; evaporation of solvent gave *IX* (11) (2.91 g, 88%).

(25R)-Cholest-5-ene-3β,26-diol (X). A mixture of *IX* (2.71 g, 4.2 mmol), hydrazine hydrochloride (3.25 g), and 85% hydrazine hydrate (16.7 g) in diethylene glycol (50 ml) was heated at 135 C for 90 min. After the addition of KOH (8.2 g), the resulting mixture was heated at 220 C for 3.5 h with removal of water by distillation (9). After cooling to room temperature, the mixture was diluted with water and extracted with $CHCl_3$. The extracts were washed with water, dried over Na_2SO_4, and evaporated to dryness. The crude product was subjected to MPLC on silica gel with 40% ethyl acetate in hexanes; evaporation of solvent gave *X* (1.21 g, 71%).

Scheme 2

(25R)-Cholest-4-ene-3-one-26-ol (XI). A solution of *X* (0.84 g, 2.0 mmol) in 55 ml toluene and 2 ml 1-methyl-4-piperidone was refluxed under a Dean-Stark trap until 3 ml distillate had collected. After cooling to RT, aluminum isopropoxide (0.92 g, 2.3 mmol) was added. The mixture was refluxed for 8 h, cooled, diluted with 100 ml diethyl ether, washed twice with 50 ml each time with 1 M HCl and twice with 75 ml saturated NaCl each time, and dried over Na_2SO_4. The crude product was purified by MPLC on silica gel with ethyl acetate in hexanes. Evaporation of solvent afforded *XI* (12) (0.72 g, 86%).

(25R)-Cholest-4-ene-3-one-26-oic acid (Δ⁴-dafachronic acid, II). Jones reagent (0.4 ml, 0.50 mmol) was added slowly and dropwise to a stirred solution of *XI* (40.7 mg, 0.10 mmol) in acetone (8 ml) at 0 C. After stirring for 1 h, the reaction was quenched by addition of excess 2-propanol, and the product was extracted with diethyl ether. The organic phase was washed with 10% $NaHCO_3$, dried over Na_2SO_4, filtered and concentrated. The crude material was purified by MPLC on silica gel with 40% ethyl acetate in hexanes, and evaporation of solvent gave *II* (3) (38 mg, 90%).

(25R)-3β,26-Bis(tert-butyldimethylsilyloxy)cholest-5-ene (XII). Similar to the procedure for *VIII*, *X* (0.652 g, 1.6 mmol) was diprotected with *tert*-butyl dimethylsilylchloride (0.94 g, 6.40 mmol) and DBU (0.90 g, 6.07 mmol) in 20 ml THF at RT for 16 h to afford *XII* (900 mg, 88%).

(25R)-Cholest-5-ene-3β-tert-butyldimethylsilyloxy-26-ol (XIII). To the solution of *XII* (1.1 g, 1.7 mmol) in 10 ml each methanol and dichloromethane was added portionwise camphorsulphonic acid (0.236 g, 1.0 mmol). After 0.5 h, the reaction was quenched with saturated $NaHCO_3$ and extracted twice with 5 ml ethyl acetate, dried over Na_2SO_4, concentrated, and purified by MPLC on silica column with 8% ethyl acetate in hexanes. Evaporation of the solvent afforded *XIII* (0.304 g, 82%).

(25R)-Cholest-5-ene-3β-tert-butyldimethylsilyloxy-26-al (XIV). A stirred solution of *XIII* (0.114 g, 0.22 mmol) in 20 ml dichloromethane was treated with Dess Martin periodinane (0.25 g, 0.46 mmol) at RT for 4 h. The reaction was quenched with saturated $Na_2S_2O_3$ solution, diluted with water, and extracted with ethyl acetate. The organic layers were dried over Na_2SO_4, and the crude product was subjected to MPLC on silica gel with ethyl acetate in hexanes. Evaporation of solvent afforded *XIV* (0.102 g, 90%).

(25R)-Cholest-5-ene-3β-tert-butyldimethylsilyloxy-26-oic acid (XV). A solution of *XIV* (18 mg, 0.035 mmol) in 4 ml methanol was treated with 2-methyl 2-butene (40 μl, 0.35 mmol) and stirred for 5 min at RT. A freshly prepared solution of 1.25 M $NaClO_2$ in 20% NaH_2PO_4 (6.4 μl, 0.070 mmol) was added, and the reaction was stirred at RT for 1 h. After completion of reaction, water (4 ml) was added, and the product was extracted twice into 20 ml ethyl acetate, which was dried over Na_2SO_4. Purification by MPLC silica gel with 12% ethyl acetate in hexanes and removal of solvent afforded *XV* (16 mg, 86%).

(25R)-Cholest-5-ene-3β-ol-26-oic acid (XVI). A solution of *XV* (16 mg, 0.030 mmol) in 3 ml THF was treated with 1 ml HF (48% in H_2O) at 0 C, slowly brought to RT, and stirred for 4 h. After completion, the reaction mixture was extracted with ether (2 × 20 ml), dried over Na_2SO_4, concentrated, and purified by MPLC on silica gel with 30% ethyl acetate in hexanes. Evaporation of solvent afforded *XV* (10 mg, 80%).

(25R)-Cholest-5-ene-3-one-26-oic acid (Δ⁵-dafachronic acid, III). A solution of *XVI* (10 mg, 0.024 mmol) in 2 ml 1:1 dimethylsulfoxide/THF was stirred at RT with iodoxybenzoic acid (53 mg, 0.192 mmol) for 8 h. The reaction was quenched with saturated NH_4Cl solution, diluted with water, extracted into ethyl acetate, dried over Na_2SO_4, and purified by MPLC on silica gel with 20% ethyl acetate in hexanes. Evaporation of solvent afforded *III* (8 mg, 80%).

Scheme 3

(25R)-3β,26-Bisacetoxy-cholest-5-ene (XVII). To a solution of *X* (1.31 g, 3.25 mmol) in dry pyridine (15 ml) was added acetic anhydride (3 ml) and *N*,*N*-dimethylaminopyridine (4 mg, 0.027 mmol). The mixture was stirred at RT under nitrogen for 18 h. Water (5 ml) was added, and the product was extracted with ethyl acetate. The organic layer was washed with $NaHCO_3$ and water and then dried over Na_2SO_4 and purified by MPLC on silica gel with 6% ethyl acetate in hexanes. Evaporation of solvent gave *XVII* (13, 14) (1.21 g, 76%).

(25R)-3β,26-Bisacetoxy-cholest-5-ene-7-one (XVIII). To a solution of *XVII* (0.405 g, 0.89 mmol) in dichloromethane (50 ml) was added 3,5-dimethyl pyrazole (1.46 g, 15.3 mmol) and chromium trioxide (1.52 g, 15.3 mmol) at −20 C. The reaction mixture was slowly brought to RT, stirred overnight, filtered through a small plug of silica gel, and extracted into ethyl acetate (200 ml), which was washed with water (3 × 50 ml) and dried over Na_2SO_4. The crude product (0.82 g) was subjected to MPLC on silica gel with ethyl acetate in hexanes, and evaporation of solvent gave *XVIII* (0.41 g, 90%).

(25R)-3β,26-Bisacetoxy-cholest-7-one (XIX). To a solution of *XVIII* (0.40 g, 0.86 mmol) in ethyl acetate (50 ml) was added Pd/C (20 mg, 5% wt/wt). A hydrogen-filled balloon (1 atm) was connected over the solution, which was stirred overnight. The reaction mixture was filtered through a pad of silica gel, which was washed with ethyl acetate, and the combined solutions were concentrated and subjected to MPLC on silica gel with 20% ethyl acetate in hexanes. Evaporation of the solvent afforded *XIX* (0.38 g, 95%).

(25R)-3β,26-Bisacetoxy-cholest-7α-ol (XX). To a stirred solution of *XIX* (0.33 g, 0.67 mmol) in THF (15 ml) was added L-Selectride (Aldrich; 1 M, 0.82 ml, 0.82 mmol) at −78 C. The resulting solution was stirred at −78 C for 3 h and quenched with 3.5 ml saturated $NaHCO_3$ and 10 ml 35% aqueous H_2O_2. The solution was stirred at RT for 1 h,

and the aqueous phase was extracted with ethyl acetate (3 × 15 ml). The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The residue was purified by MPLC on silica gel with 20% ethyl acetate in hexanes; evaporation of the solvent gave *XX* (0.26 g, 78%), a single diastereomer by NMR.

(25R)-3β,26-Bisacetoxy-cholest-7-ene (XXI). To a stirred solution of *XX* (0.18 g, 0.38 mmol) in benzene (5 ml) was added Burgess reagent (0.18 g, 0.76 mmol) at RT. The solution was refluxed for 3 h, quenched with water, extracted with ethyl acetate (20 × 3 ml), dried over Na_2SO_4, and subjected to MPLC on silica gel with 10% ethyl acetate in hexanes. Evaporation of the solvent gave *XXI* (0.15 g, 86%).

(25R)-Cholest-7-ene-3β,26-diol (XXII). To a stirred solution of *XXI* (0.15 g, 0.33 mmol) in 5 ml methanol was added KOH (100 mg), and the solution was refluxed for 4 h. The reaction mixture was neutralized with 1 N HCl, and methanol was removed under reduced pressure. The residue was and extracted with ethyl acetate, which was dried with Na_2SO_4 and purified by MPLC with ethyl acetate in hexanes. Evaporation of the solvent afforded *XXII* (0.12 g, 90%).

(25R)-Cholest-7-ene-3-one-26-al (XXIII). A stirred solution of *XXII* (0.15 g, 0.37 mmol) in 20 ml dichloromethane was treated with Dess Martin periodinane (0.33 g, 0.78 mmol) at RT for 4 h. The reaction was quenched with saturated $Na_2S_2O_3$ solution, diluted with water, and extracted with ethyl acetate. The organic layers were dried over Na_2SO_4, and the crude product was subjected to MPLC on silica gel with ethyl acetate in hexanes. Evaporation of solvent afforded *XXIII* (0.12 g, 81%).

(25R)-Cholest-7-ene-3-one-26-oic acid (Δ^7-dafachronic acid, I). A solution of *XXIII* (51 mg, 0.126 mmol) in 4 ml methanol was treated with 2-methyl 2-butene (90 μl, 1.27 mmol) and stirred for 5 min at RT. A freshly prepared solution of 1.25 M $NaClO_2$ in 20% NaH_2PO_4 (24 μl, 0.026 mmol) was added, and the reaction was stirred at RT for 1 h. After completion of reaction, water (10 ml) was added, and the product was extracted twice into 30 ml ethyl acetate, which was dried over Na_2SO_4. Purification by MPLC silica gel with 24% ethyl acetate in hexanes afforded *I* (42 mg, 80%).

Scheme 4: Δ^7 series

(25R,S)-Cholest-7-ene-3-one-26-al (R/S-XXIII). A solution of *XXIII* (0.14 g, 0.35 mmol) in 5 ml THF with 1 ml DBU was stirred at RT for 48 h. Water was added, and the products were extracted with ethyl acetate, which was dried over Na_2SO_4. The crude product was purified by MPLC on silica gel with ethyl acetate in hexanes, and evaporation of solvent afforded *R/S-XXIII* (0.13 g, 93%).

(25R,S)-Cholest-7-ene-3,26-diol (R/S-XXII). To a stirred solution of *R/S-XXIII* (0.13 g, 0.33 mmol) in 6 ml methanol at 0 C was added $NaBH_4$ (0.026 g, 0.67 mmol), and the reaction was brought slowly to RT. After 1 h, water was added, and the product was extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and purified by MPLC on silica gel with ethyl acetate in hexanes; evaporation of solvent afforded *R/S-XXII* (0.12 g, 92%). The stereochemistry of the 3-hydroxyl, which was lost in subsequent steps, was not determined.

(25S)-Cholest-7-ene-26-acetoxy-3-ol (XXIV). A solution of *R/S-XXII* (0.12 g, 0.30 mmol) in 8 ml $CHCl_3$ was stirred at RT for 5 min and treated with *Pseudomonas cepacia* lipase (10 mg) and then 5 min later with vinyl acetate (0.5 ml) (15). The reaction mixture was stirred at RT until thin-layer chromatography indicated that approximately 50% of starting material was converted to product (48 h). The reaction mixture was filtered and purified by MPLC on silica gel with ethyl acetate in hexanes; evaporation of solvent afforded *XXIV* (0.055 g, 42% based on starting material, 84% of theoretical).

(25S)-Cholest-7-ene-3,26-diol (S-XXII). A methanolic solution of *XXIV* (0.052 g, 0.117 mmol) was refluxed with KOH under conditions described for *XXII*, yielding *S-XXII* (0.045 g, 92%).

(25S)-Cholest-7-ene-3-one-26-al (S-XXIII). The *S-XXII* (0.045 g 0.11 mmol) in dichloromethane was oxidized to *S-XXIII* with Dess Martin periodinane according to the procedure for *XXIII* (0.035 g, 92%).

(25S)-Cholest-7-ene-3-one-26-oic acid (Δ^7-dafachronic acid, S-I). A solution of *S-XXIII* (32 mg, 0.08 mmol) in 5 ml methanol was treated with 2-methyl 2-butene (56 μl, 0.8 mmol) and stirred for 5 min at RT. A freshly prepared solution of 1.25 M $NaClO_2$ in 20% NaH_2PO_4 (14.4 μl, 0.16 mmol) was added, and the reaction was stirred at RT for 1 h. Water (10 ml) was added, and the product was extracted twice into 30 ml ethyl acetate, which was dried over Na_2SO_4. Purification by MPLC silica gel with 24% ethyl acetate in hexanes afforded *S-I* (4) (0.030 mg, 90%).

Scheme 4: Δ^4 series

(25R)-Cholest-4-ene-3-one-26-al (XXV). Compound *XI* (0.10 g, 0.024 mmol) was oxidized with Dess Martin periodinane as described for other alcohols above (0.082 g, 82%).

(25R,S)-Cholest-4-ene-3-one-26-al (R/S-XXV). The aldehyde *XXV* (0.082 g 0.20mmol) was epimerized with DBU as described for *XXIII* to give *R/S-XXV* (0.064 g, 78%).

(25R,S)-Cholest-4-ene-3,26-diol (XXVI). Aldehydes *R/S-XXV* (0.064 g 0.164 mmol) were reduced with $NaBH_4$ in methanol similar to the preparation of *R/S-XXII* (0.052 g, 80%). The stereochemistry of the 3-hydroxyl, which was lost in subsequent steps, was not determined.

(25S)-Cholest-4-ene-26-acetoxy-3-ol (XXVII). Stereospecific acetylation of *XXVI* (0.064 g, 0.158 mmol) with lipase was performed as with *R/S-XXII* to afford *XXVII* (0.052 g, 80%).

(25S)-Cholest-4-ene-3,26-diol (S-XXVI). Saponification of *XXVII* (0.060 g, 0.135 mmol) with methanolic KOH was performed as with *S-XXII* to afford *S-XXVI* (0.48 g, 87%).

(25S)-Cholest-4-ene-3-one-26-oic acid (Δ^4-dafachronic acid, S-II). Jones reagent (0.44 ml, 0.55 mmol) was added slowly and dropwise to a stirred solution of *S-XXVI* (40.7 mg, 0.10 mmol) in acetone (8 ml) at 0 C. After stirring for 1 h, the reaction was quenched by addition of excess 2-propanol, and the product was extracted with diethyl ether. The organic phase was washed with 10% $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated. The crude material was purified by MPLC on silica gel with 40% ethyl acetate in hexanes, and evaporation of solvent gave *II* (8) (42 mg, 90%).

Scheme 4: Δ^5 series

(25RS)-Cholest-5-ene-3β-tert-butyldimethylsilyloxy-26-al (R/S-XIV). The aldehyde *XIV* (0.102 g, 0.19 mmol) was epimerized with DBU as described for *XXIII* to give *R/S-XIV* (0.096 g, 94%).

(25RS)-Cholest-5-ene-3β-tert-butyldimethylsilyloxy-26-ol (R/S-XIII). To a stirred solution of *R/S-XIV* (0.094 g, 0.18 mmol) in 3 ml methanol at 0 C was added $NaBH_4$ (0.008 g, 0.22 mmol), and the reaction was brought slowly to RT. After 1 h, water was added, and the product was extracted with ethyl acetate. The organic layer dried over Na_2SO_4 and purified by MPLC on silica gel with ethyl acetate in hexanes. Evaporation of solvent afforded *R/S-XIII* (84 mg, 82% based on starting material recovery). The stereochemistry of the 3-hydroxyl, which was lost in subsequent steps, was not determined.

(25S)-Cholesta-3β-tert-butyldimethylsilyloxy-26-acetoxy-5-ene (XXVIII). Enzymatic acetylation of *R/S-XIII* (82 mg, 0.16 mmol) using the pro-

cedure for *R/S-XXII* afforded *XXVIII* (35 mg, 40% based on starting material, 80% of theoretical).

(25S)-Cholesta-5-ene-3β-tert-butyldimethylsilyloxy-26-ol (S-XIII). Saponification of *S-XIII* (0.018 g, 0.032 mmol) with methanolic KOH according to the procedure for *XXIV* afforded *S-XIII* (0.015 g, 90%).

(25S)-Cholest-5-ene-3β-tert-butyldimethylsilyloxy-26-oic acid (S-XV). Two-step oxidation of *S-XIII* (12 mg, 0.023 mmol) as described for conversion of *XIII* to *XV* afforded *S-XV* (9 mg, 73%).

(25S)-Cholest-5-ene-3β-ol-26-oic acid (S-XVI). A solution of *S-XVI* (8 mg, 0.015 mmol) in 4 ml THF was stirred with tetrabutyl ammonium fluoride (1 M, 0.1 ml) at RT for 8 h. The reaction was quenched with saturated NH_4Cl solution, diluted with water, extracted into ethyl acetate, dried over Na_2SO_4, and purified by MPLC on silica gel with ethyl acetate in hexanes to afford *S-XVI* (6 mg, 95%).

(25S)-Cholest-5-ene-3-keto-26-acid (S-III). A solution of *S-XVI* (4 mg, 0.009 mmol) in 4 ml 1:1 dimethylsulfoxide/THF was stirred at RT with iodoxybenzoic acid (201 mg, 0.072 mmol) for 8 h. The reaction was quenched with saturated NH_4Cl solution, diluted with water, extracted into ethyl acetate, dried over Na_2SO_4, and purified by MPLC on silica gel with ethyl acetate in hexanes to afford *S-III* (3.6 mg, 90%).

Scheme 5

(25R)-5α-Cholest-26-ol-3-one (XXIX). To a solution of *XI* (16 mg, 0.040 mmol) in 3 ml isopropanol was added Pd/$CaCO_3$ (4 mg, 25% wt/wt). A hydrogen-filled balloon (1 atm) was connected over the solution, which was stirred overnight. The reaction mixture was filtered through a pad of silica gel, which was washed with ethyl acetate, and the combined solutions were concentrated and subjected to MPLC on silica gel with 16% ethyl acetate in hexanes. Evaporation of solvent afforded *XXIX* (14 mg, 87%).

(25R)-5α-Cholest-3-one-26-oic acid (IV). Jones reagent (0.12 ml, 0.15 mmol) was added slowly and dropwise to a stirred solution of *XXIX* (12 mg, 0.029 mmol) in 4 ml acetone at 0 C. After stirring for 1 h, the reaction was quenched by addition of excess 2-propanol, and the product was extracted with diethyl ether. The organic phase was washed with 10% $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated. The crude material was purified by MPLC on silica gel with 36% ethyl acetate in hexanes. Evaporation of solvent afforded *IV* (42 mg, 90%).

(25R)-5β-Cholest-3-one-26-oic acid (V). A solution of *II* (5 mg, 0.012 mmol) in 5 ml methanol was treated with Pd/C (2 mg, 40% wt/wt) and ammonium formate (12 mg, 0.24 mmol), and the solution was refluxed for 6 h (16). The reaction mixture was filtered through a pad of silica gel, which was washed with ethyl acetate, and the combined solutions were concentrated and subjected to MPLC on silica gel with 20% ethyl acetate in hexanes. Evaporation of solvent afforded *V* (4 mg, 80%).

(25S)-5α-Cholest-3,26-diol (S-XXX). A solution of *S-XXVI* (12 mg, 0.029 mmol) in 2 ml acetonitrile and 1 ml isopropanol with Pd/$CaCO_3$ (2 mg, 16% wt/wt) was stirred overnight under 1 atm of hydrogen, which was maintained by a hydrogen-filled balloon. The reaction mixture was filtered through a pad of silica gel, which was washed with ethyl acetate, and the combined solutions were concentrated and subjected to MPLC on silica gel with 20% ethyl acetate in hexanes. Evaporation of solvent afforded *S-XXX* (10 mg, 82%).

(25S)-5α-Cholest-3-one-26-oic acid (S-IV). Jones reagent (0.028 ml, 0.035 mmol) was added slowly and dropwise to a stirred solution of *S-XXX* (3 mg, 0.007 mmol) in 2 ml acetone at 0 C. After stirring for 1 h, the reaction was quenched by addition of excess 2-propanol, and the product was extracted with diethyl ether. The organic phase was washed with 10% $NaHCO_3$, dried over Na_2SO_4, filtered, and concentrated. The crude material was purified by MPLC on silica gel with 32% ethyl acetate in hexanes. Evaporation of solvent afforded *S-IV* (8) (2 mg, 65%).

(25S)-5β-Cholest-3-one-26-oic acid (S-V). To a solution of *S-II* (5 mg, 0.012 mmol) in 5 ml methanol was treated with Pd/C (2 mg, 40% wt/wt) and ammonium formate (15 mg, 0.29 mmol), and the solution was refluxed for 6 h (16). The reaction mixture was filtered through a pad of silica gel, which was washed with ethyl acetate, and the combined solutions were concentrated and subjected to MPLC on silica gel with 20% ethyl acetate in hexanes. Evaporation of solvent afforded *S-V* (4 mg, 80%).

NMR and optical rotation data, as well as schemes, are found in the supplemental data file.

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