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A Nuclear Receptor Ligand-based Probe Enables Temporal Control of *C. elegans* Development

Joshua C. Judkins, M. Sc., Parag Mahanti, M. Sc., Jacob Hoffman, Isaiah Yim, Prof. Dr. Adam Antebi, and Prof. Dr. rer. nat. Frank C. Schroeder*

Boyce Thompson Institute and Department of Chemistry and Chemical Biology Cornell University
1 Tower Road, Ithaca, New York 14853 (USA)

Max Planck Institute for Biology of Ageing Joseph Stelzmann Str. 9b, D-50931 Cologne
(Germany)

Abstract

C. elegans development and lifespan are controlled by the nuclear hormone receptor DAF-12, an important model for vertebrate vitamin D and liver-X receptors. Similar to its mammalian homologs, DAF-12 function is regulated by bile acid-like steroidal ligands, the dafachronic acids; however, tools for investigating their biosynthesis and function in vivo are lacking. We report a flexible synthesis for DAF-12 ligands and masked ligand derivatives that enable precise temporal control of DAF-12 function. For ligand masking, we introduce photocleavable amides of 5-methoxy-N-methyl-2-nitroaniline (MMNA). MMNA-masked ligands are bioavailable and after incorporation into the worm can be used to trigger expression of DAF-12 target genes and initiate development from dauer larvae to adults by brief, innocuous UV-irradiation. In-vivo release of DAF-12 ligands and other small-molecule signals using MMNA-based probes will enable functional studies with precise spatial and temporal resolution.

Keywords

small-molecule signaling; synthesis; steroid hormones; photolysis

Nuclear Hormone Receptors (NHRs) play a central role in metazoan development and metabolism.^[1] Many NHRs are regulated by small-molecule ligands, and extensive studies of mammalian vitamin-D receptor (VDR),^[2] peroxisome proliferator-activated receptors (PPAR),^[3] and estrogen receptors (ER)^[4] have shown that binding of a variety of natural and synthetic ligands can lead to different gene expression profiles.^[2, 4]

The nematode *Caenorhabditis elegans* is a particularly useful model organism for the study of NHR biology because of its short lifecycle and close homology of many signaling pathways to those in higher organisms.^[5] In *C. elegans*, the NHR DAF-12 is a central regulator of life history, triggering reproductive development under favorable conditions or developmental arrest at the long lived dauer stage when environmental conditions are

*fs31@cornell.edu Homepage: <http://bti.cornell.edu/schroeder>.

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unfavorable (Figure 1A).^[5b, 6] In addition, DAF-12 plays a major role in the regulation of adult lifespan in response to signals from the reproductive system.^[7] DAF-12 is a homolog of VDR, farnesoid-X (FXR) and liver-X (LXR) receptors, and, like its vertebrate counterparts, DAF-12 function is regulated by steroidal ligands.^[5b] These DAF-12-activating steroids, collectively called the dafachronic acids (DAs), feature a carboxylated side chain and varying functionalization of the steroidal A- and B-rings.^[8] We recently showed that previous hypotheses about the *endogenous* ligands of DAF-12 and their biosynthesis must be revised, and that the most prevalent endogenous DAs include unexpected ¹-desaturation and 3 α -OH hydroxylation (dafa#3 and hyda#1, respectively, see Figure 1B).^[8]

Several lines of evidence indicate that the DAs serve different functions at different time points in the worm's lifecycle^[5, 7f] and that biosynthesis of DAs occurs via different routes in different tissues.^[8b, 9] These findings further increase the significance of *C. elegans* as a model for vertebrate NHR biology and associated small-molecule signaling pathways; however, appropriate tools for investigating DA biosynthesis and function *in vivo* are lacking. Further advancement of the field will require development of strategies that enable tissue-specific liberation of small molecules in live *C. elegans* with precise temporal control. Here we introduce 5-methoxy-*N*-methyl-2-nitroaniline (MMNA) amides as photocleavable masking groups that are easy to attach, biocompatible, and enable targeted release of different DAF-12 ligands and putative biosynthetic precursors *in vivo*. In addition, we report a short, flexible synthesis of the new DAF-12 ligands (for syntheses of the previously known dafa#1 and derivatives, see^[10]).

Analysis of the substitution patterns of the identified DAF-12 ligands led us to choose lithocholic acid (**1**) and chenodeoxycholic acid **3** as inexpensive starting materials (Figure 1B). LiAlH₄ reduction of **1** followed by Ag₂CO₃ oxidation and sidechain extension via Horner-Wadsworth-Emmons (HWE) reaction produced intermediate **2**, which after basic hydrolysis, diastereoselective hydrogenation with (*S*)-Ru(OAc)₂[H₈-BINAP],^[10b] and dehydrogenation with IBX:NMO yielded dafa#2 in only 6 steps and 32% overall yield. Subsequent dissolving metal reduction of the ⁴-double bond in dafa#2 using Li/NH₃ produced the putative biosynthetic precursor dafa#4 (See Supporting Information). Synthesis of the ⁷-unsaturated dafa#1 and dafa#3 followed a similar sequence, but required protecting the 7 α -hydroxy group as a benzyloxymethyl ether (BOM) during the initial part of the synthesis, deprotection of which was concomitant with the dissolving metal reduction of the ⁴-unsaturated intermediate **4** to achieve *trans*-decalin configuration of the steroid core (Figure 1B). Ag₂CO₃-Celite oxidation yielded ketoaldehyde **5**, which after HWE reaction, Burgess elimination^[11] and late-stage diastereoselective reduction with (*S*)-Ru(OAc)₂[H₈-BINAP] yielded (2*S*)-dafa#1 in 10 steps and 12% overall yield. Subsequent treatment with IBX/TFA introduced the ¹-double bond with high regioselectivity to yield dafa#3, whereas reduction with K-selectride produced the 3 α -OH-substituted hyda#1. A prior synthesis^[10b] of dafa#1 used the same approach for introduction of the chiral side chain, but at a much earlier stage.

To develop inactive, photocleavable derivatives of DAF-12 ligands or biosynthetic precursors that could be incorporated *in vivo*, the properties of 5-methoxy-*N*-methyl-2-

nitroaniline (MMNA) amides were investigated (Figure 2). Compared to nitroindole and nitrodehydroquinoline derivatives previously used for masking carboxylic acids,^[12] MMNA offers similar quantum efficiency (Table S1), but is commercially available and less prone to undesired oxidative degradation. Irradiation at 365 nm of MMNA-masked (*RS*)-2-methylundecanoic acid or MMNA-masked dafa#4, a likely intermediate in DAF-12 ligand biosynthesis,^[8b] resulted in nearly quantitative conversion into the free acids, as indicated by NMR spectroscopic analysis of the reaction mixtures (Figures 2, S2). We then investigated whether MMNA-protected DAF-12 ligands are biocompatible and stable under physiological conditions, and whether these derivatives could be used to liberate DAF-12 ligands or precursors *in vivo*. For these studies *daf-9(dh6)* mutant worms were used, which are defective in the CYP450 enzyme that catalyzes the last step in DAF-12 ligand biosynthesis.^[6b, 8a, 13] As a result, *daf-9(dh6)* mutant worms lack endogenous DAF-12 ligands and constitutively arrest development as long-lived dauer larvae, unless synthetic ligands are added that trigger resumption of development to normal adult worms (“dauer rescue”).^[8a]

The *in vivo* stability of MMNA-protected DAF-12 ligands was tested by placing arrested *daf-9(dh6)* worms in growth media containing 1 μ M MMNA-dafa#1 or MMNA-dafa#4. All treated worms remained arrested for the entire duration of the experiment (2 days), indicating that MMNA-protected dafachronic acids do not act as DAF-12 ligands and are not hydrolyzed to form free DAF-12 ligands. Worms treated with MMNA-dafa#1 remained viable as demonstrated by resumption of development upon UV-irradiation of the plates (Figure S4). To test whether MMNA derivatives are taken up by the worms and can be used to generate active DAF-12 ligand inside the worm, we treated arrested *daf-9(dh6)* worms with MMNA-masked dafa#1, washed them extensively, and transferred them to untreated agar plates (Figure 3A). Treated worms did not develop and remained arrested during the entire experiment (up to 6 days), even when using high concentrations of MMNA-masked ligand. However, brief irradiation (365 nm, 90 sec) of arrested *daf-9(dh6)* worms up to 4 days after treatment with MMNA-dafa#1 consistently triggered resumption of development to the adult stage. These results show that (1) MMNA-masked steroids are readily taken up by *C. elegans*, (2) the MMNA derivatives are non-toxic and retained in the worm body for several days, and (3) brief, innocuous irradiation is sufficient to unmask biologically relevant quantities of active ligand inside the worm. To confirm that irradiation of MMNA-dafa#1-treated worms triggers development in fact via activation of DAF-12, we used *daf-9(dh6)* animals that express green fluorescent protein (GFP) under the control of the promoter of a highly conserved microRNA, *mir-84*, a known target of DAF-12 involved in lifespan regulation (Figure 3B).^[7c, 9a] *mir-84* is strongly expressed in two rows of cells along the sides of the worm body (the seam cells), and thus ligand-based activation of DAF-12 in *pmir-84::GFP* worms leads to green fluorescence in the seam cells.^[7c, 9a] As shown in Figure 3, irradiation of *daf-9(dh6)* (*pmir-84::GFP*) worms treated with MMNA-dafa#1 produced strong fluorescence in the seam cells, similar to what is observed for treatment with unmodified dafa#1 (also see Figures S5 and S6).

These results demonstrate that MMNA-masked derivatives can be used to deliver functional nuclear hormone receptor ligands inside the worm body with precise temporal control, and

present a first example for light-triggered in vivo release of endogenous small molecule signals in *C. elegans*. MMNA-protected DAF-12 ligands and ligand precursors provide new tools for the study of the signaling cascades up- and downstream of DAF-12,^[5a, 7f, 14] which are of great interest for further developing *C. elegans* as a model for aging in higher animals.^[7c, 15] Steroids that are structurally related to the DAs, for example common bile acids,^[16] may play a role in mammalian lifespan regulation and should be similarly amenable to MMNA derivatization. In combination with tissue-specific gene knock-outs, localized irradiation of animals treated with MMNA-masked signaling molecules will enable the study of tissue-specific biosyntheses and functions, one of the major challenges in understanding small-molecule signaling in *C. elegans* and other metazoans.^[5b] Lastly, we here report an improved synthesis that provides more direct access to newly identified and known DAF-12 ligands than previously reported routes.^[10]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Under favorable conditions, cholesterol is converted into ligands of the nuclear hormone receptor DAF-12, triggering development to adult worms. Under unfavorable conditions, ligand biosynthesis is abolished, DAF-12 binds to its co-repressor DIN-1, and larvae arrest at the long lived dauer stage. B) Synthesis of DAF-12 ligands (dafa#1-dafa#3 and hyda#1, see www.smid-db.org for nomenclature) and derived photocleavable probes. a. LiAlH_4 , reflux; b. Ag_2CO_3 -Celite, reflux; c. triethyl-2-phosphonopropionate; LiCl , DIEA; d. LiOH ; e. $(S)\text{-Ru}(\text{OAc})_2(\text{H}_8\text{-BINAP})$, H_2 ; f. IBX:NMO; g. TMSCHN_2 ; h. BOM-Cl , DIEA; i. IBX, TFA; j. Li , NH_3 ; k. Burgess reagent, reflux; l. K-selectride; m. 1: $\text{C}_2\text{O}_2\text{Cl}_2$, DMF; 2: 5-methoxy-*N*-methyl-2-nitroaniline, pyridine. A NOESY spectrum was used to confirm *trans* configuration of the double bond in **2** (see Figure S1).

Figure 2.

A) Irradiation of MMNA-dafa#4 at 365 nm yielded dafa#4 and byproducts **7** and **8**. B) UV-Vis spectra of MMNA-masking (*RS*)-2-methylundecanoic acid with increasing irradiation at 365 nm in $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (3:1). C) Aromatic region of ^1H NMR spectra (600 MHz, CDCl_3) of MMNA-2-methylundecanoic acid before (top) and after (bottom) irradiation, showing signals of MMNA-dafa#4, **7** and **8**. Note that MMNA-dafa#4 exist as a mixture of three rotamers in a ratio of 1:0.83:0.32, resulting in three sets of ^1H NMR signals (see Figure S3). D) Aliphatic regions of ^1H NMR spectra (600 MHz, CDCl_3) of dafa#4, MMNA-dafa#4 (mixture of rotamers), and irradiated MMNA-dafa#4, indicating quantitative conversion of MMNA-dafa#4 into dafa#4.

Figure 3.

In vivo release of dafa#1 activates DAF-12 and triggers development in ligand-deficient *daf-9(dh6)* mutant worms. A) Simplified scheme for assay. B) Left, positive control: addition of synthetic dafa#1 to arrested *daf-9(dh6)(pmir-84::GFP)* worms triggers seam cell fluorescence (white arrows) and development. Center: worms treated with MMNA-dafa#1 remain arrested, even after several days, and no seam cell fluorescence is observed. Right: worms treated with MMNA-dafa#1 initiated development upon irradiation up to 4 days after treatment and show strong GFP expression in the seam cells (white arrows). See Supporting Information for experimental details.