Optogenetics Hot Paper

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A Photocleavable Masked Nuclear-Receptor Ligand Enables Temporal Control of *C. elegans* Development**

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

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 (PPARs),[3] and estrogen receptors (ERs)[4] have shown that the 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 the close homology of many of its signaling pathways to those of higher organisms.[5] In *C. elegans*, the NHR DAF-12 is a central regulator of life history; it triggers reproductive development under favorable conditions or developmental arrest at the long-lived dauer stage when environmental conditions are unfavorable (Figure 1 A).[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 homologue of the 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 concerning the endogenous ligands of DAF-12 and their biosynthesis must be revised, and that the most prevalent endogenous DAs include unexpected Δ^1-desaturation and 3α-OH hydroxylation (dafa#3 and hyda#1, respectively, see Figure 1 B).[8]

Several lines of evidence indicate that DAs serve different functions at different time points in the worms' lifecycle[5,7f] and that the biosynthesis of DAs occurs through 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 the development of strategies that enable the tissue-specific liberation of small molecules in live *C. elegans* with precise temporal control. Herein, 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 1 B). $LiAlH_4$ reduction of **1** followed by Ag_2CO_3 oxidation and side-chain extension through Horner–Wadsworth–Emmons (HWE) reaction produced intermediate **2**, which after basic hydrolysis, diastereoselective hydrogenation with (*S*)-[$Ru(OAc)_2(H_8$-BINAP)],[10b] and dehydrogenation with IBX·NMO yielded dafa#2 in only 6 steps and 32% overall yield. Subsequent dissolving metal reduction of the Δ^4-double bond in dafa#2 by using Li/NH_3 produced the putative biosynthetic precursor dafa#4 (see the Supporting Information). The synthesis of the Δ^7-unsaturated dafa#1 and

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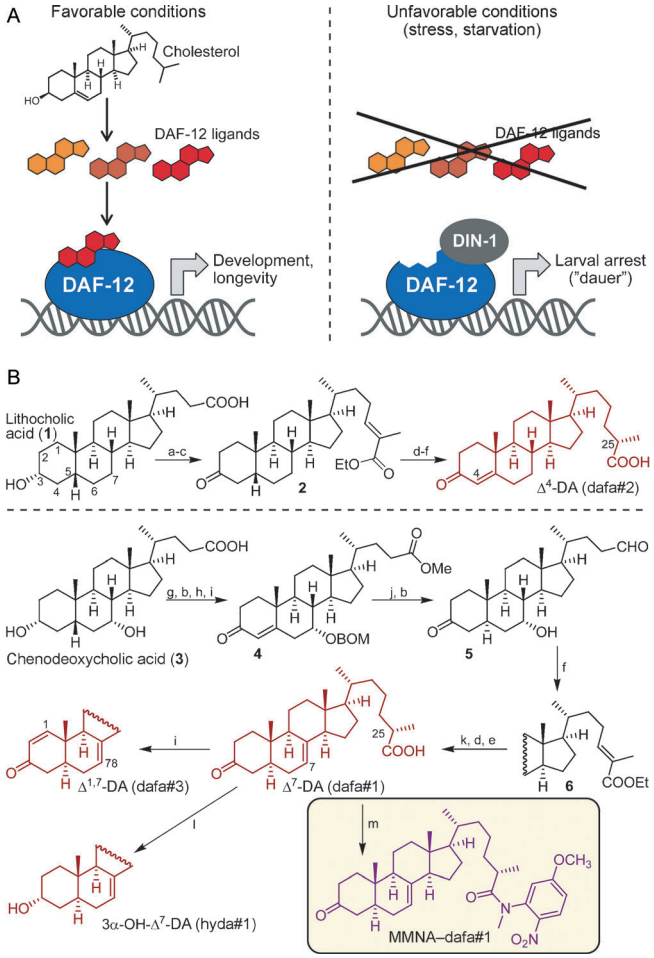

Figure 1. A) Under favorable conditions, cholesterol is converted into ligands of the nuclear hormone receptor DAF-12, thereby triggering development into adult worms. Under unfavorable conditions, ligand biosynthesis is abolished, DAF-12 binds to its corepressor DIN-1, and larvae are arrested at the 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 MMNA-masked ligands. a) $LiAlH_4$, reflux; b) Ag_2CO_3-Celite, reflux; c) triethyl-2-phosphonopropionate; LiCl, DIEA; d) LiOH; e) (*S*)-[Ru(OAc)$_2$(H_8-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: $C_2O_2Cl_2$, DMF; 2: 5-methoxy-*N*-methyl-2-nitroaniline, pyridine. A NOESY spectrum was used to confirm the *trans* configuration of the double bond in **2** (see Figure S1). DIEA = *N*,*N*-diisopropylethylamine, BINAP = 2,2′-bis(diphenylphosphanyl)-1,1′-dinaphthyl, IBX = 2-iodoxybenzoic acid, NMO = *N*-methylmorpholine *N*-oxide, $TMSCHN_2$ = trimethylsilyldiazomethane, TFA = trifluoroacetic acid, DMF = *N*,*N*-dimethylformamide.

dafa#3 followed a similar sequence but required protection of the 7α-hydroxy group as a benzyloxymethyl ether (BOM) during the initial part of the synthesis. Deprotection was concomitant with the dissolving metal reduction of the Δ^4-unsaturated intermediate **4** to achieve a *trans*-decalin configuration of the steroid core (Figure 1B). Oxidation with Ag_2CO_3 adsorbed on Celite (Ag_2CO_3-Celite) yielded ketoaldehyde **5**, which after HWE reaction, Burgess elimination,[11] and late-stage diastereoselective reduction with (*S*)-[Ru(OAc)$_2$(H_8-BINAP)] yielded (25*S*)-dafa#1 in 10 steps and 12% overall yield. Subsequent treatment with IBX and TFA introduced the Δ^1-double bond with high regioselectivity to yield dafa#3, whereas reduction with K-selectride produced the 3α-OH-substituted hyda#1. A prior synthesis[10b] of dafa#1 used the same approach for the 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 the nitroindole and nitrodehydroquinoline derivatives previously used for masking carboxylic acids,[12] MMNA offers similar quantum efficiency (Table S1 in the Supporting Information) but is commercially available and less prone to undesired oxidative degradation. MMNA-masked variants of dafa#1, dafa#4 (a likely intermediate in DAF-12 ligand biosynthesis),[8b] and a simple 2-methyl-branched carboxylic acid, (RS)-2-methylundecanoic acid, were prepared. Irradiation at 365 nm resulted in nearly quantitative conversion into the free acids, as indicated by NMR spectroscopic analysis of

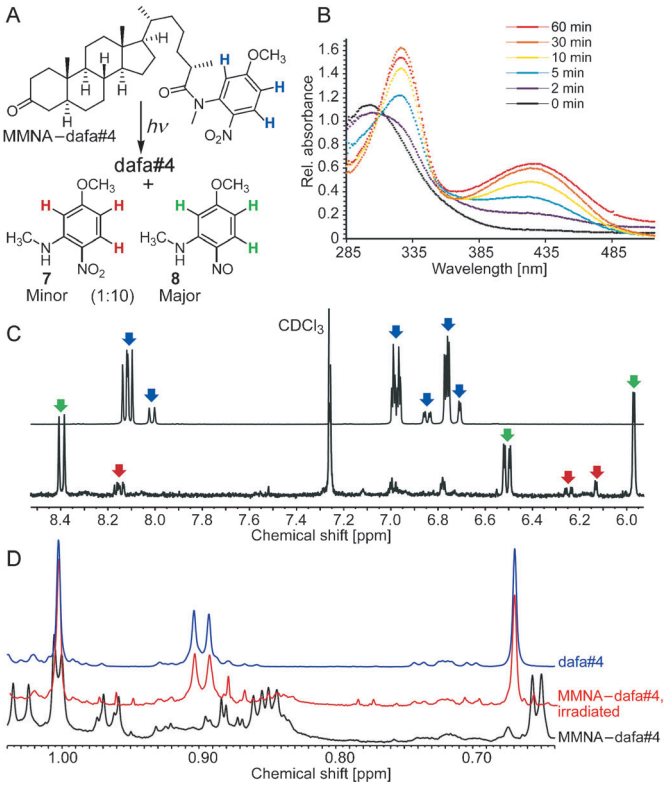

Figure 2. A) Irradiation of MMNA–dafa#4 at 365 nm yielded dafa#4 and byproducts **7** and **8**. B) UV/Vis spectra of MMNA-masked (*RS*)-2-methylundecanoic acid with increasing irradiation at 365 nm in CH_3CN/H_2O (3:1). C) Aromatic region of the ^{1}H NMR spectra (600 MHz, $CDCl_3$) of MMNA–2-methylundecanoic acid before (top) and after (bottom) irradiation, showing signals for MMNA–2-methylundecanoic acid (blue arrows), **7** (red arrows), and **8** (green arrows). Note that MMNA–2-methylundecanoic acid exists as a mixture of three rotamers in a ratio of 1:0.83:0.32, thus resulting in three sets of ^{1}H NMR signals (see Figure S3). D) Aliphatic regions of the ^{1}H 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.

the reaction mixtures (Figure 2 and Figure S2 in the Supporting Information). We then investigated whether the 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 into normal adult worms ("dauer rescue").[8a]

The in vivo stability of the MMNA-protected DAF-12 ligands was tested by placing arrested *daf-9(dh6)* worms in growth media containing 1 μM MMNA–dafa#1 or MMNA–dafa#4. All treated worms remained arrested for the entire duration of the experiment (2 days), thus indicating that the 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 the 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 3 A). 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 nontoxic and are 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 the irradiation of MMNA–dafa#1-treated worms in fact triggers development through 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 DAF-12 target involved in lifespan regulation (Figure 3 B).[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 that observed following treatment with unmodified dafa#1 (also see Figures S5 and S6).

These results demonstrate that MMNA-masked derivatives can be used to deliver functional NHR ligands inside the worm body with precise temporal control, and present a first example for the 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 upstream 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 knockouts, 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 report herein an improved synthesis that provides more direct access to newly identified and known DAF-12 ligands than previously reported routes.[10]

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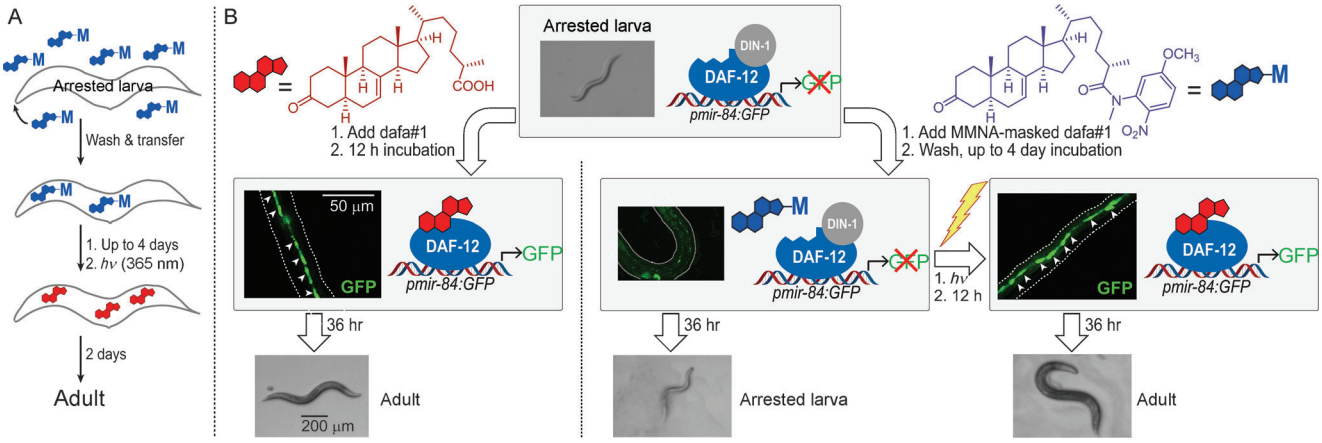

Figure 3. The in vivo release of dafa#1 activates DAF-12 and triggers development in ligand-deficient *daf-9(dh6)* mutant worms. A) Simplified Scheme for assay. B) Left, positive control: addition of synthetic dafa#1 to arrested *daf-9(dh6)(pmir-84:GFP)* worms triggers seam cell fluorescence (white arrows) 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).

Keywords: amides · gene expression · photolysis · small-molecule signaling · steroid hormones

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