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# Optimization of α-Acylaminoketone Ecdysone Agonists for Control of Gene Expression

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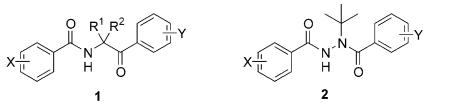
**Abstract**—Fifteen new α-acylaminoketones were prepared by four different routes in an initial effort to optimize the potency of these compounds as ecdysone agonists. The compounds were assayed in mammalian cells expressing the ecdysone receptors from *Bombyx mori* (BmEcR) and *Choristoneura fumiferana* (CfEcR) for their ability to cause expression of a reporter gene downstream of an ecdysone response element. A new α-acylaminoketone was identified which had activity equal to that of the standard dibenzoylhydrazine ecdysone agonist GS™-E in the assay based on CfEcR.


We recently reported the discovery of novel ecdysone agonist **1a** (Fig. 1), an α-acylaminoketone derived synthetically from 1-aminocyclopentane-1-carboxylic acid.[1] Non-steroidal ecdysone agonists, notably GS™-E (**2b**), are important components in systems to control gene expression[2,3] based on engineered ecdysone receptors.[4–10] We were therefore interested in discovering more potent analogues of **1a**.

Our previous SAR study of α-acylaminoketones of general structure **1** had shown that **1a**, in which $R^1$ and $R^2$ form a cyclopentane ring, was significantly more potent than **1b** (Table 1), in which $R^1$ and $R^2$ are both ethyl groups. Thus our initial efforts were directed towards exploration of the effect of ring size on potency. Two previously described synthetic routes[1] were used (Scheme 1). In Method A, α,α-disubstituted amino acids **3** were converted to the corresponding azlactones **4** which were opened with *N*,*O*-dimethylhydroxylamine to afford Weinreb amides **5**.[12] Weinreb amides **5** were reacted with 3,5-dimethylphenylmagnesium bromide to afford new α-acylaminoketones **1d–f**. Desired cyclopropane and cyclobutane products **1d** and **1e** were the major components of the crude products derived from **5d** and **5e** isolated in 68 and 66% yields, respectively; however, similar treatment of **5a** and **5f** gave less satisfactory results and more extensive purification was required to isolate cyclopentane **1a** and cyclohexane **1f** in 40 and 28% yields, respectively.[13] Method B, in which azlactones **4** were converted into **1** in four steps via aldehydes **6**, proved more satisfactory for the preparation of **1a** and **1f** despite its greater length. Method B was also applied to the synthesis of geminal dimethyl compound **1c** and cycloheptane **1g**. All target compounds were characterized by $^1$H NMR and either $^{13}$C NMR or electrospray MS. Certain analogues were more fully characterized.[14]

The initial set of compounds **1a–g** was screened in dose response assays in two different cell lines engineered to express ecdysone receptors from lepidopteran species and reporter genes under the control of ecdysone response elements. The first assay used a previously reported HEK-293 cell-line engineered to express the ecdysone receptor from *Bombyx mori* (BmEcR) and a β-galactosidase reporter gene.[6,15] The second assay used CHO cells expressing a modified *Choristoneura fumiferana*



**1**

**1a** X = 2-Me-3-MeO, $R^1$-$R^2$ = -$(CH_2)_4$-, Y =3,5-diMe

**2**

**2a** X = 2-Me-3-MeO, Y = 3,5-diMe
**2b** X = 2-Et-3-MeO, Y = 3,5-diMe

**Figure 1.** Non-steroidal ecdysone agonists.

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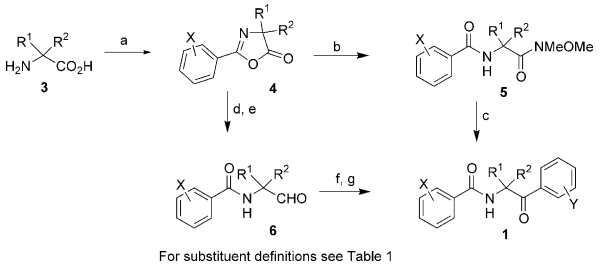
**Table 1.** Structure and transactivation assay results of α-acylaminoketone analogues **1**

| Compd | X | $R^1$, $R^2$ | Y | Synthetic method[a] | BmEcR[b] $EC_{50}$ (μM)[d]/RMFI[e] | CfEcR[c] $EC_{50}$ (μM)[d]/RMFI[e] |
|---|---|---|---|---|---|---|
| **1a** | 2-Me-3-MeO | $-(CH_2)_4-$ | 3,5-diMe | A or B | 1.87/0.85 | 1.91/0.86 |
| **1b** | 2-Me-3-MeO | Et, Et | 3,5-diMe | B | 4.53/0.61 | 6.23/0.54 |
| **1c** | 2-Me-3-MeO | Me, Me | 3,5-diMe | B | 10.00/0.17 | 41.95/0.29 |
| **1d** | 2-Me-3-MeO | $-(CH_2)_2-$ | 3,5-diMe | A | 20.36/0.22 | 33.30/0.09 |
| **1e** | 2-Me-3-MeO | $-(CH_2)_3-$ | 3,5-diMe | A | 8.52/0.69 | 10.00/0.89 |
| **1f** | 2-Me-3-MeO | $-(CH_2)_5-$ | 3,5-diMe | A or B | 1.41/0.99 | 1.11/0.96 |
| **1g** | 2-Me-3-MeO | $-(CH_2)_6-$ | 3,5-diMe | B | 1.61/0.69 | 2.97/0.88 |
| **1h** | 2-Me-3-MeO | $-(CH_2)_4-$ | 2-MeO | B | 5.16/0.70 | 6.61/0.67 |
| **1i** | 2-Me-3-MeO | $-(CH_2)_5-$ | 2-MeO | B | 0.98/0.84 | 1.03/0.71 |
| **1j** | 2-Me-3-MeO | $-(CH_2)_6-$ | 2-MeO | B | 1.94/0.78 | 3.36/0.66 |
| **1k** | 2-Me-3-MeO | $-(CH_2)_2O(CH_2)_2-$ | 3,5-diMe | A | 10.00/0.18 | 10.00/0.96 |
| **1l** | 2-Me-3-MeO | $-(CH_2)_2S(CH_2)_2-$ | 3,5-diMe | A | 1.60/0.98 | 1.80/0.85 |
| **1m** | 2-Me-3-MeO | $-(CH_2)_2SO_2(CH_2)_2-$ | 3,5-diMe | A[f] | 15.00/0.10 | 30.00/0.04 |
| **1n** | 2-Et-3-MeO | $-(CH_2)_5-$ | 3,5-diMe | C | 1.81/0.85 | 1.25/0.89 |
| **1o** | 2-Me-3,4-$OCH_2O$ | $-(CH_2)_5-$ | 3,5-diMe | C | 3.68/0.81 | 3.22/0.91 |
| **1p** | 2-Et-3,4-$OCH_2CH_2O$ | $-(CH_2)_5-$ | 3,5-diMe | C | 0.74/0.89 | 0.39/0.91 |
| **1q** | 4-Et | $-(CH_2)_5-$ | 3,5-diMe | B[g] or D | 1.51/0.63 | [h] |
| **2a** | 2-Me-3-MeO | n/a | 3,5-diMe | n/a | 0.10/1.03 | 0.28/1.02 |
| **2b** | 2-Et-3-MeO | n/a | 3,5-diMe | n/a | 0.25/1.00 | 0.41/1.00 |

[a]See Scheme 1 for Methods A and B and Scheme 2 for Method C.
[b]See ref 15 for assay protocol.
[c]See ref 16 for assay protocol.
[d]Dose affording 50% of maximum transactivation.
[e]Ratio of maximum level of gene expression of compound to maximum level of gene expression with **2b**.
[f]Prepared from **1i** by treatment with oxone in aqueous methanol.
[g]4-Ethylbenzoyl chloride (**16b**) was substituted for 3-methoxy-2-methylbenzoyl chloride in Scheme 1.
[h]Compound not tested.




**Scheme 1.** α-Acylaminoketone synthesis methods A (via **5**) and B (via **6**). (a) X-PhCOCl (2.5 equiv), pyridine, rt; (b) MeNHOMe.HCl, pyridine, $CH_2Cl_2$, rt; (c) Y-PhMgBr (4 equiv), THF, rt, 5 h; (d) $NaBH_4$, THF, rt: (e) Dess–Martin periodinane, $CH_2Cl_2$, rt; (f) Y-PhMgBr (4 equiv), THF, −70 °C → rt; (g) Dess–Martin periodinane, $CH_2Cl_2$, rt.

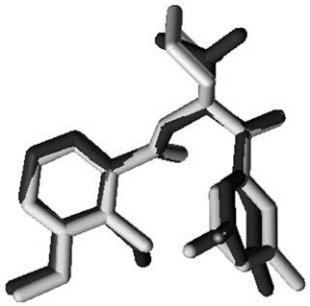


**Figure 2.** Overlap of the X-ray structures of **1f** (grey) and **2b** (black).

ecdysone receptor (CfEcR) and a luciferase reporter gene.[11,16] The results are reported in Table 1 in terms of $EC_{50}$ and maximum fold induction relative to the standard ecdysone agonist GS™-E (**2b**)[17] which was used as a positive control. Similar SAR trends were observed in both assays. Cyclohexane **1f** proved to be a more effective ligand (lower $EC_{50}$ and higher relative maximum fold induction) than either the previous lead compound, cyclopentane **1a**, or its homologue cycloheptane **1g**. Activity dropped off sharply as the ring size was decreased to cyclobutane **1e** and cyclopropane **1d**. The superior potency of the cyclohexane ring was further confirmed by synthesis and bioassay of **1h–j** in which the Y substituent is 2-methoxy rather than 3,5-dimethyl.

Next, compounds **1k–m** were prepared to explore the effect of introduction of heteroatoms into the cyclohexane ring. **1k** and **1m** which display hydrogen bond accepting ether and sulfone functionality were 9- and 27-fold less potent, respectively, than parent cyclohexane **1f**. By contrast, the more hydrophobic thioether **1l** retained most of the activity of **1f**.

Finally, we turned our attention to optimization of the substitution X on the benzamide ring of **1**. As before we were guided by the SAR of the related dibenzoylhydrazine ecdysone agonists **2** in the selection of **1n–q** as target compounds.[18] Our confidence in the validity of this analogy was bolstered by comparison of the X-ray crystal structures of **1f**[19] and **2b**.[20] The unit cell of **1f** consisted of one molecule of methanol and two molecules of the α-acylaminoketone in different conformers. Figure 2 shows the overlap of one conformer each of **1f** and **2b**. The RMSD between the backbone C–C(=O)–N–C–C(=O)–C atoms of **1f** and the C–C(=O)–N–N–C(=O)–C atoms of **2b** in these conformers was 0.081 Å.

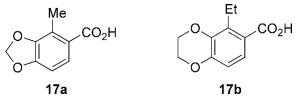
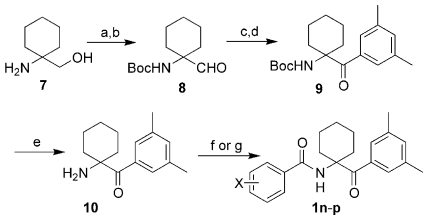
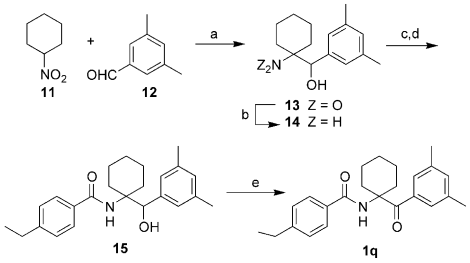



**Figure 3.** Acids used for preparation of **1o** and **1p**.




**Scheme 2.** Method C for synthesis of α-acylaminoketones. (a) $(Boc)_2O$, THF, rt, 18 h; (b) Dess–Martin periodinane, $CH_2Cl_2$, rt, 6 h; (c) 3,5-diMe-PhMgBr, THF, −70 °C, 1 h; (d) Dess–Martin periodinane, $CH_2Cl_2$, rt, 20 h; (e) $CF_3CO_2H$, $CH_2Cl_2$, rt, 1 h; (f) X-PhCOCl (**16**), pyridine, $CH_2Cl_2$; (g) X-PhCO$_2$H (**17**), EDC.HCl, DMAP, *i*-$Pr_2NEt$, $CH_2Cl_2$, THF, rt, 24 h.




**Scheme 3.** Method D for synthesis of α-acylaminoketones. (a) NaOMe, MeOH, 5 °C > rt, 16 h; (b) Zn, conc HCl, MeOH, 5 °C → rt, 16 h; (c) 4-Et-PhCOCl (**16b**, 2.2 equiv), pyridine, THF, rt, 16 h; (d) 10% aq NaOH, MeOH, THF, rt, 16 h; (e) Dess–Martin periodinane, $CH_2Cl_2$, rt, 6 h.

In the second conformer of **1f** (not shown) the torsional angle between the 3-methoxy-2-methyl substituted benzene ring was rotated by ca. 180° about the bond to the carbonyl carbon.[21]

While Method B would have been a workable synthetic approach to **1n–q**, we sought methods that would allow incorporation of the benzamide ring later in the synthesis. Two methods were developed. Firstly, in Method C (Scheme 2) the known aminoalcohol **7**[22] was protected as its Boc derivative and oxidized with Dess–Martin periodinane to afford aldehyde **8** in 89% yield. Reaction of **8** with 3,5-dimethylphenylmagnesium bromide afforded a secondary alcohol which was oxidized with the Dess–Martin periodinane to give ketone **9** in 73% yield. TFA mediated removal of the Boc protecting group from **9** liberated the aminoketone **10** in 71% yield. Treatment of **10** with 2-ethyl-3-methoxybenzoyl chloride (**16a**) afforded **1n** while carbodiimide coupling with acids **17a**[23] and **17b**[24] (Fig. 3) gave the desired α-acylaminoketones **1o** and **1p**, respectively.[25,26]

In Method D, nitrocyclohexane (**11**) was reacted with benzaldehyde **12** under literature conditions[27] to afford **13** in 14% yield (Scheme 3). Nitroaldol adduct **13** was reduced to aminoalcohol **14** in 85% yield. Treatment of **14** with one equivalent of 4-ethylbenzoyl chloride (**16b**) gave a mixture of unreacted **14**, mono- and diacylated products. Thus, **14** was treated overnight with an excess of **16b** to ensure complete *N*-acylation and the reaction mixture was immediately saponified to cleave any *O*-benzoylated products and afford clean alcohol **15**. Oxidation of **15** with Dess–Martin periodinane afforded the desired α-acylaminoketone **1q** in 16% yield from **14**.

Compound **1n**, with an *ortho*-ethyl group on the benzamide ring, was slightly less active than *ortho*-methoxy compound **1f**. The 2-methyl-3,4-methylenedioxy compound **1o** was less active than **1f**; however, the 2-ethyl-3,4-ethylenedioxy analogue **1p** proved to be the most potent α-acyalminoketone ligand tested in both the BmEcR and CfEcR based assays. Its potency was equal to that of the standard ligand GS™-E (**2b**) in the CfEcR based assay.

We have described the synthesis of fifteen new α-acylaminoketones of general structure **1** by four different routes and their activity as ligands for the control of gene expression in systems based on lepidopteran EcRs. We have determined the crystal structure of **1f**, a representative analogue. Compound **1p** is the most potent ecdysone agonist of general structure **1** described to date.

## Acknowledgements

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14. Compound **1f**: Mp 161–163 °C. $^1$H NMR ($CDCl_3$, 300 MHz) δ 1.2–1.9 (8H), 1.96 (s, 3H), 2.05 (m, 2H), 2.31 (s, 6H), 3.80 (s, 3H), 6.25 (br s, 1H), 6.75 (d, $J = 7.6$ Hz, 1H), 6.85 (d, $J = 8.2$ Hz, 1H), 7.08 (s, 1H), 7.14 (m, 1H), 7.51 (s, 2H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 11.9, 21.3, 21.7, 25.1, 35.4, 55.6, 64.0, 111.3, 118.2, 125.0, 125.8, 126.5, 133.0, 137.2, 137.3, 137.7, 158.0, 168.7, 203.1. IR ($CDCl_3$) 1680, 2939 $cm^{-1}$. MS (ESI-ve ion) *m*/*z* 380 (M-1). Anal. calcd for $C_{24}H_{29}NO_3$: C, 75.96; H, 7.70; N, 3.69. Found: C, 75.77; H, 7.94; N, 3.83.
15. A bulk transformed HEK-293 cell line, created as described in ref 6, was provided by Gage and Suhr. Dilution cloning was used to isolate individual clones. Clone Z3 was selected using 450 μg $mL^{-1}$ G418 and 100 ng $mL^{-1}$ puromycin. Cells were trypsinized and diluted to a concentration of $2.5 \times 10^4$ cells $mL^{-1}$. Cell suspension 100 μL was placed in each well of a 96 well plate (Dynex, 14-245-182) and incubated at 37 °C under 5% $CO_2$ for 24 h. Ligand stock solutions were prepared (10 mM in DMSO) and diluted 100-fold in media. 50 μL of diluted ligand solution was added to each well. The final concentration of DMSO was maintained at 0.03% in both controls and treatments. β-Galactosidase reporter gene expression was measured 48 h after treatment of the cells using Gal Screen™ bioluminescent reporter gene assay system from Tropix (GSY1000). Luminescence was detected at room temperature using a Dynex MLX microtiter plate luminometer. Fold inductions were calculated by dividing relative light units (RLU) in ligand treated cells with RLU in DMSO treated cells.
16. CHO cells were transiently transfected with transcription cassettes for GAL4 DBD (1-147) *Cf*EcR(DEF) and for VP16 TAD βRXR-*Lm*USP ECH9 controlled by ubiquitously active cellular promoters (PGK and EF-1α, respectively) on a single plasmid. Stably transfected cells were selected by Zeocin resistance. Individually isolated CHO cell clones were transiently transfected with a GAL4 RE-luciferase reporter (pFR Luc). 27-63 clone was selected using Hygromycin. Cells were trypsinized and diluted to a concentration of $2.5 \times 10^4$ cells mL. 100 μL of cell suspension was placed in each well of a 96-well plate and incubated at 37 °C under 5% $CO_2$ for 24 h. Ligand stock solutions were prepared in DMSO and diluted 300-fold for all treatments. Dose–response testing consisted of 8 concentrations ranging from 33 to 0.01 μM. Luciferase reporter gene expression was measured 48 h after cell treatment using Bright-Glo™ Luciferase Assay System from Promega (E2650). Luminescence was detected at room temperature using a Dynex MLX microtiter plate luminometer. $EC_{50}$s were calculated from dose response data using a three parameter logistic model.
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