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ARTICLE *in* ORGANIC LETTERS · MAY 2004

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2004
Vol. 6, No. 9
1409−1412

Facile Synthesis of CIDs: Biotinylated Estrone Oximes Efficiently Heterodimerize Estrogen Receptor and Streptavidin Proteins in Yeast Three Hybrid Systems

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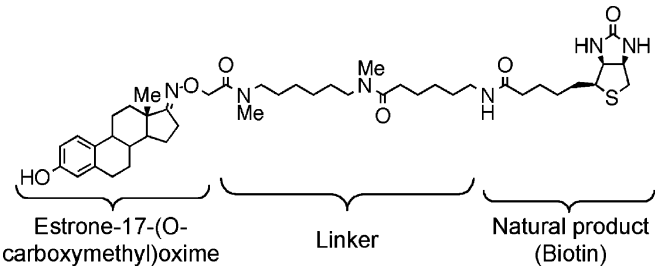
Received February 11, 2004

ABSTRACT



We synthesized estrone oximes as chemical inducers of protein heterodimerization (CIDs). Estrone-17-(*O*-carboxymethyl)oxime coupled to biotinamidocaproic acid via *N*,*N*′-dimethylhexane-1,6-diamine efficiently heterodimerizes estrogen receptors (ERs) and streptavidin Y43A in yeast three hybrid systems, activating gene expression over 100-fold at 10 μM. Related hexane-1,6-diamine and estradiol-6-(*O*-carboxymethyl)-oxime derivatives were ineffective CIDs due to low affinity for ERs when bound to streptavidin. Estrone oximes bind ERs with submicromolar affinity and effectively display small molecules to target proteins expressed in yeast.

Chemical inducers of protein dimerization (CIDs) are powerful molecular tools for probing diverse biological processes. CIDs have been used to investigate signal transduction pathways,[1−8] regulate gene expression,[9−12] control protein secretion,[13] activate protein splicing,[14] manipulate enzyme activity,[15−17] and identify protein targets of small molecules.[18−20]

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10.1021/ol0497537 CCC: $27.50
Published on Web 04/01/2004

The identification of protein targets of natural products and other biologically active small molecules has been traditionally pursued by affinity chromatography methods. In this approach, a small molecule of interest is typically immobilized on an insoluble support and treated with cellular extracts. Noninteracting proteins can be washed from the support, whereas specifically interacting proteins are retained, enriched, and can be eluted with a specific competitor. Peptide fragments of eluted proteins are analyzed by sequencing, and this sequence information can be matched with databases to identify genes encoding putative protein targets. However, given the low abundance and low stability of many proteins, this strategy can be quite technically challenging.

CIDs can be used to identify protein targets of natural products by activating gene expression in living yeast cells.[18,19] In these yeast three hybrid systems, the natural product is covalently linked to a cell-permeable ligand such as the steroid dexamethasone.[18,19] A receptor such as the dexamethasone-binding glucocorticoid receptor is expressed in yeast fused to a DNA binding domain (DBD). This DBD (e.g., the bacterial LexA protein) anchors the receptor on specific DNA sites of a reporter gene in the nucleus of yeast cells. Addition of a cell-permeable CID results in binding of the ligand element to the receptor and displays the linked natural product to other target proteins expressed in yeast. Genetic libraries (e.g., cDNA libraries) encoding tens of thousands of potential target proteins each fused to a transcriptional activation domain (AD, e.g., the B42 protein) can be introduced and coexpressed in these recombinant yeast. Because each yeast cell in theory expresses a unique protein member of the library, yeast cells can be rapidly screened to identify putative protein targets of the natural product. Binding of a target−AD fusion protein to the natural product displayed by the receptor−DBD fusion protein reconstitutes a functional transcription factor by positioning the AD in proximity of specific DNA sites of a reporter gene. This transcription factor in turn recruits the cellular transcriptional machinery to DNA to activate expression of a reporter gene typically encoding a readily detected enzyme such as β-galactosidase.[21,22] A major potential advantage of this genetic approach over affinity chromatography is the ability to immediately sequence specific genes encoding putative protein targets without requiring purification of unstable protein products.

We report here a simple two-pot synthesis of a novel, highly active, and cell-permeable CID (**1** Figure 1) comprising an estrone (*O*-carboxymethyl)oxime linked to biotin.

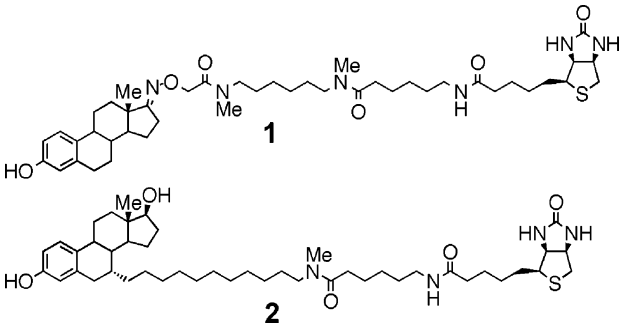

Figure 1. Structures of novel (**1**) and previously reported (**2**) CIDs that efficiently heterodimerize ER and SA proteins.

Estrone (**7**) is a high affinity ligand of estrogen receptor (ER) proteins, and the appended natural product biotin provides a simple model of complex natural products with unknown protein targets. CID **1** resembles our previously reported biotinylated 7α-substituted β-estradiol derivative **2**, an efficient heterodimerizer of ERs with the biotin-binding protein streptavidin (SA) in yeast three hybrid systems (Figure 2).[23]

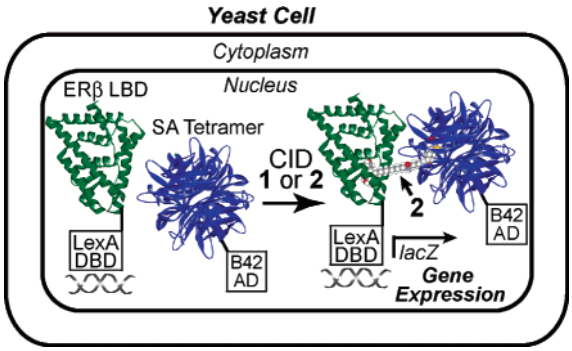

Figure 2. Schematic of the ER/SA yeast three hybrid system showing a previously reported[23] model of a ternary ER-**2**-SA complex. Heterodimerization of ER-LexA and SA-B42 by CIDs **1** or **2** activates expression of the *lacZ* (β-galactosidase) reporter gene.

Although **2** is an exceptionally active CID in yeast,[23] the routine use of 7α-substituted β-estradiol derivatives as CIDs is limited by the lengthy 14-step synthesis required to prepare these compounds. [23,24]

As a dramatically streamlined alternative, a two-pot synthesis of CID **1** and control compounds **3**−**5** (Figure 3), is shown in Scheme 1. (*O*-Carboxymethyl)oxime derivatives of estrone (**7**) and 6-ketoestradiol (**9**)[25] were prepared in quantitative yield from treatment with aminooxyacetic acid

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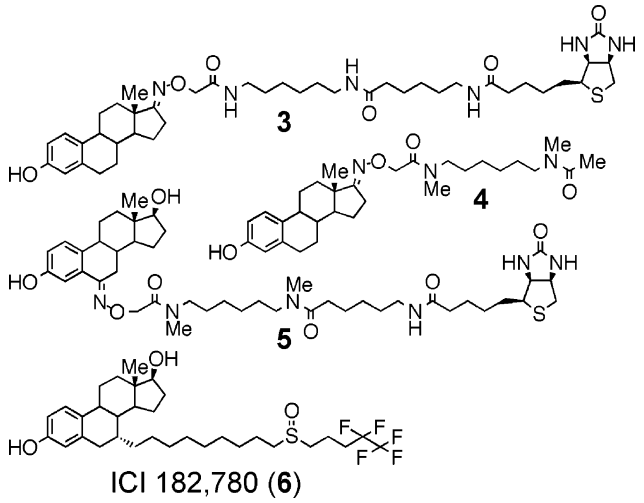
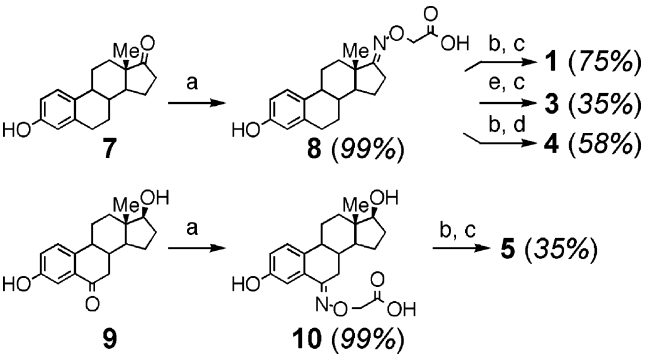


Figure 3. Structures of control compounds.

hydrochloride in pyridine.[26,27] The resulting carboxylic acids were converted to *N*-hydroxysuccinimidyl esters in situ and treated with excess 1,6-hexanediamine or *N*,*N*′-dimethyl-1,6-hexanediamine, and the resultant amine product was subjected to a second acylation step to afford compounds **1** and **3**−**5** in good to moderate overall yields.

Scheme 1. Two-Pot Synthesis of Compounds **1** and **3**−**5**[a]



[a] Reagents and conditions: (a) $H_2NOCH_2CO_2H$·HCl, pyridine; (b) DCC, *N*-hydroxysuccinimide (NHS), $MeHN(CH_2)_6NHMe$, CH_2Cl_2; (c) D-biotinamidocaproic acid NHS ester, DIEA, CH_2Cl_2, MeOH; (d) acetic acid NHS ester, DIEA, CH_2Cl_2; (e) DCC, NHS, $H_2N(CH_2)_6NH_2$, CH_2Cl_2.

To assess the effectiveness of **1** and related analogues **3** and **5** as CIDs, these compounds were compared with CID **2** in our ER/SA yeast three hybrid system.[23] In these experiments, the bacterial LexA DBD[28] was expressed as a fusion protein linked to either the ERα or ERβ ligand binding domains (LBDs). The B42 AD[28] was similarly coexpressed fused to the C-terminus of SA Y43A.[23] This SA mutant exhibits low toxicity in yeast[23] and binds biotin with an affinity of $K_d \sim 100$ pM.[29] Yeast were also transformed with the commercially available reporter vector pSH18-34 (Invitrogen). This reporter contains the *lacZ* gene encoding β-galactosidase under the control of four dimeric LexA DNA binding sites. The nonbiotinylated analogue **4** and the antiestrogen ICI 182,780 (**6**)[30] were also examined in these assays as negative control compounds. As shown in Figure 4, compounds **1** and **2** strongly activated dose-dependent

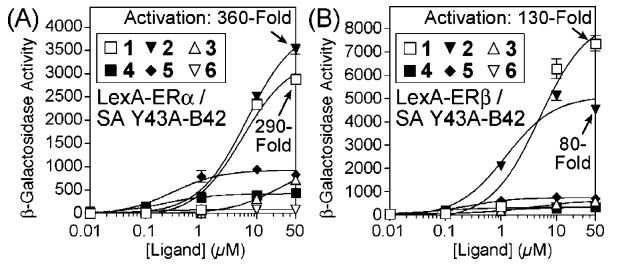


Figure 4. Dose−response curves from yeast three hybrid assays. Panel A: ERα assays. Panel B: ERβ assays. Fold activation = observed β-Gal activity/β-Gal activity without ligand.

reporter gene expression in yeast three hybrid systems expressing ERα or ERβ fusion proteins. At concentrations of 10 and 50 μM in the ERα assay, the previously reported CID **2** was only slightly more active than CID **1**, with both compounds enhancing reporter gene expression by ∼300-fold above basal levels (50 μM). In yeast expressing ERβ, CID **1** proved to be the most active compound at 10 and 50 μM, activating gene expression 130-fold above basal levels (50 μM), albeit with a 4-fold reduction in potency compared with **2** (Table 1). Surprisingly, the structurally similar

Table 1. Compilation of EC_{50} and IC_{50} Values Quantified by Nonlinear Regression Analysis of the Data Shown in Figures 4 and 6[a]

ligand	Y3H ERα + SA	Y3H ERβ + SA	FP ERα	FP ERα + SA
1	5600 ± 2200	4900 ± 2200	240 ± 130	720 ± 330
2	6600 ± 1500	1200 ± 470	16 ± 10	330 ± 220
3			290 ± 170	2100 ± 1200
4			200 ± 80	370 ± 160
5			1400 ± 450	4200 ± 1600
6			14 ± 10	22 ± 10
7			2.8 ± 1.5	3.4 ± 1.1

[a] Y3H: yeast three hybrid EC_{50} values (nM). FP: competition fluorescence polarization IC_{50} values (nM). Errors correspond to 95% confidence intervals.

analogues **3** and **5** were much less effective CIDs, possibly due in part for **5** to lower stability of this electron-rich aromatic oxime in relatively acidic yeast media (pH = 3 to 4). Furthermore, as expected, the control compounds lacking biotin (**4**, **6**) were essentially inactive in these assays.

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To evaluate the specificity of CID **1**, competition and omission experiments were employed (Figure 5). These

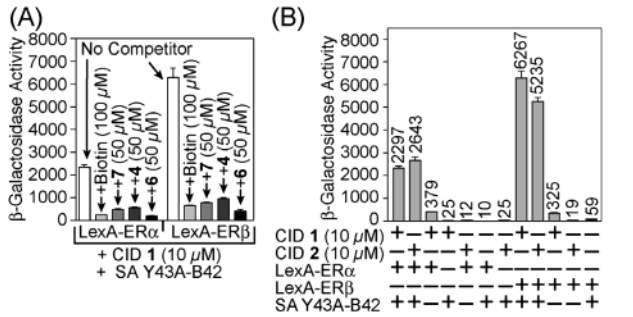

Figure 5. Yeast three hybrid competition experiments (panel A) and omission experiments (panel B).

experiments confirmed that specific ER or SA ligands blocked reporter gene expression. Omission experiments confirmed the importance of each protein component of the yeast three-hybrid system and compared the influence of CIDs **1** and **2** on elements of this system. These experiments revealed that both the ER and SA proteins were required for maximal activation of gene expression. However, CID **1** promoted some one-hybrid (ER-dependent) activity not observed (<1%) with CID **2** (Figure 5, panel B). These differences in one-hybrid activity likely relate to subtle changes in ER conformation induced upon CID binding. The one-hybrid activity promoted by CID **1** (10 μM) comprised 16% of the total activity in the ERα assay but only 5% of the total activity in the ERβ assay. This lower one-hybrid activity in the ERβ assay provided a 20-fold dynamic range for activation of gene expression by SA Y43A, suggesting that ERβ is the best platform for the display of estrone-linked small molecules to target proteins in yeast three hybrid systems.

To understand the dramatic differences in activity observed for CIDs **1** and **2** compared with the structurally similar **3** and **5**, the affinities of compounds for recombinant ERα protein were quantified with competition fluorescence polarization (FP) assays (Figure 6). These experiments compared competition IC_{50} values for ERα both in the absence and presence of equimolar recombinant SA protein to

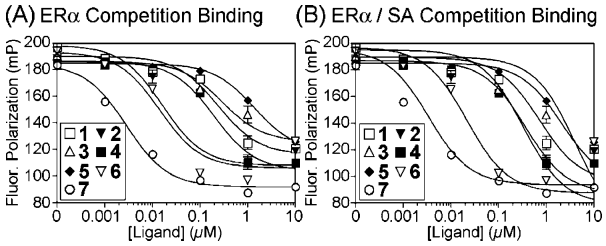

Figure 6. Fluorescence polarization assays with recombinant ERα protein. Panel A: Competition binding experiments with ERα. Panel B. Competition binding experiments with ERα and equimolar streptavidin (SA) protein.

examine the effects of this protein on binding of ligands to ERα. As summarized in Table 1, 7α-substituted estradiol derivatives such as **2** and **6** exhibited the highest affinities for ERα (IC_{50} values <20 nM). However, added recombinant SA protein, which binds biotin irreversibly with an affinity of ~100 fM,[31] reduced the affinity of CID **2** for ERα by 21-fold (ERα/SA IC_{50} (**2**) = 330 nM). In contrast, although CID **1** bound more weakly to ERα alone (ERα IC_{50} (**1**) = 240 nM), the addition of SA reduced the affinity for ERα by only 3-fold (ERα/SA IC_{50} (**2**) = 720 nM). The similar affinities of SA-bound **1** and SA-bound **2** for ERα (within 2-fold) explain why these compounds exhibit similar maximal activities and potencies in the yeast three-hybrid assays. In contrast, in the presence of SA the affinities of **3** (rigidified by secondary amides) and **5** were substantially lower for ERα, dramatically reducing their activities as CIDs.

(*O*-Carboxymethyl)oximes derived from aliphatic ketones are typically highly stable under physiological conditions. Correspondingly, CID **1** showed no significant decomposition to estrone (**7**) even after 16 h in relatively acidic (pH = 3 to 4) yeast media (HPLC analysis provided in the Supporting Information). These results demonstrate that readily synthesized estrone oximes can provide efficient tools for the ER-mediated display of small molecules to target proteins.

Acknowledgment. We thank the American Cancer Society (RSG-02-025-01) and National Institutes of Health (R01-CA83831) for financial support.

Supporting Information Available: Experimental procedures, characterization data for new compounds, and HPLC assays of the stability of CID **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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