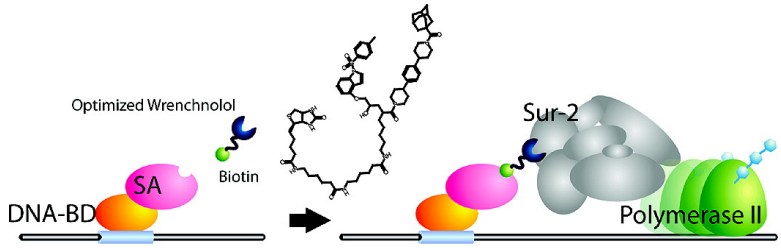
Article

Wrenchnolol Derivative Optimized for Gene Activation in Cells

Dongju Jung, Hiroki Shimogawa, Youngjoo Kwon, Qian Mao,
Shin-ichi Sato, Shinji Kamisuki, Hideo Kigoshi, and Motonari Uesugi

J. Am. Chem. Soc., **2009**, 131 (13), 4774-4782• DOI: 10.1021/ja900669k • Publication Date (Web): 16 March 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S ARTICLES

Published on Web 03/16/2009

Wrenchnolol Derivative Optimized for Gene Activation in Cells

Dongju Jung,[†,‡] Hiroki Shimogawa,[†] Youngjoo Kwon,[‡,||] Qian Mao,[‡] Shin-ichi Sato,[†,‡] Shinji Kamisuki,[†] Hideo Kigoshi,[*,§] and Motonari Uesugi[*,†,‡]

Institutes for Chemical Research and for Integrated Cell-Material Sciences and PRESTO/JST, Kyoto University, Uji, Kyoto 611-0011, Japan, The Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, and Department of Chemistry, University of Tsukuba, Tsukuba, Ibaraki 305-8571, Japan

Received January 27, 2009; E-mail: uesugi@scl.kyoto-u.ac.jp (M.U.); kigoshi@chem.tsukuba.ac.jp (H.K.)

Abstract: Naturally occurring transcription factors usually have two independent domains, a DNA-binding domain and an activation domain. In designing a synthetic small molecule that mimics a transcription factor, each of the two domains needs to be replaced by small-molecule counterparts. Results of the present study show that derivatives of wrenchnolol, a synthetic molecule that interacts with Sur-2 coactivator, serve as activation modules and stimulate gene transcription in vitro and in cells when tethered to a DNA-binding molecule. Thirteen derivatives of wrenchnolol were chemically synthesized and tested for their ability to activate transcription in vitro and in cells. When tethered to the GAL4 DNA-binding domain, one derivative increased transcription of a GAL4-responsive reporter gene in cells 9-fold. This optimized derivative also induced up to 45% myogenesis of C2C12 cells when tethered to the DNA-binding domain of myogenic transcription factor MyoD. This optimized derivative may serve as a starting point for designing biological tools or components of fully synthetic transcription factors that permit selective up-regulation of genes.

Introduction

Transcription factors are pivotal regulators of gene expression,[1] and small molecules that modulate their function have profound effects in living organisms.[2,3] An excellent example is small molecule-modulation of nuclear hormone receptors, in which endogenous or exogenous ligands bind to ligand-binding domains of the receptors and negatively or positively regulate gene activation. The human genome encodes ≈2600 DNA-binding proteins,[4] including 48 nuclear receptors.[5] Most of these DNA-binding proteins are likely to be transcription factors, but many lack domains that are inherently controlled by small molecules.

One approach to exogenous control of transcription factors is to use small organic molecules that mimic a part of, or even the entire, transcription factor. Naturally occurring transcription factors usually have two independent domains, a DNA-binding domain that provides gene selectivity to the transcription factor and an activation domain that stimulates transcription by contacting coactivator proteins. A number of efforts have been made to use small molecules to mimic these two domains. For example, pyrole-imidazole hairpin polyamides bind sequence-specifically to DNA with high affinity comparable to that of transcription factors.[6,7] Custom-designed, cell-permeable polyamides inhibit specific gene transcription in cultured mammalian cells by blocking the interactions between DNA and particular transcription factors.[8−11]

Small-molecule mimetics of activation domains have also been developed. By screening of a library of 100 000 hexameric peptoids, Kodadek and co-workers discovered cell-permeable peptoids that bind to mammalian coactivator CBP (CREB-binding protein).[12] The peptoid activation domain, conjugated with a DNA-binding hairpin polyamide, increased expression of a luciferase reporter gene 5-fold in HeLa cells, and selectively up-regulated expression of 45 genes at least 3-fold among ∼46 000 transcripts.[13] Mapp and co-workers discovered low-molecular-weight, organic activators from a library of isoxazolidine derivatives.[14] Although their target proteins remain unknown, these simple small molecules were able to enhance

[†] Kyoto University
[‡] Baylor College of Medicine
[§] University of Tsukuba
[||] Current address: College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea.

(1) Orphanides, G.; Reinberg, D. *Cell* **2002**, *108*, 439–451.
(2) Rosen, E. D.; Walkey, C. J.; Puigserver, P.; Spiegelman, B. M. *Genes Dev.* **2000**, *14*, 1293–1307.
(3) Issaeva, N.; Bozko, P.; Enge, M.; Protopopova, M.; Verhoef, L. G.; Masucci, M.; Pramanik, A.; Selivanova, G. *Nat. Med.* **2004**, *10*, 1321–1328.
(4) Babu, M. M.; Luscombe, N. M.; Aravind, L.; Gerstein, M.; Teichmann, S. A. *Curr. Opin. Struct. Biol.* **2004**, *14*, 283–291.
(5) , *Cell* **1999**, *97*, 161–163.
(6) Dervan, P. B.; Burli, R. W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 688–693.
(7) Gottesfeld, J. M.; Turner, J. M.; Dervan, P. B. *Gene Expr.* **2000**, *9*, 77–91.
(8) Olenyuk, B. Z.; Zhang, G. J.; Klco, J. M.; Nickols, N. G.; Kaelin, W. G., Jr.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16768–16773.
(9) Nickols, N. G.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10418–10423.
(10) Burnett, R.; Melander, C.; Puckett, J. W.; Son, L. S.; Wells, R. D.; Dervan, P. B.; Gottesfeld, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11497–11502.
(11) Takahashi, T.; et al. *Chem. Biol.* **2008**, *15*, 829–841.
(12) Liu, B.; Alluri, P. G.; Yu, P.; Kodadek, T. *J. Am. Chem. Soc.* **2005**, *127*, 8254–8255.
(13) Xiao, X.; Yu, P.; Lim, H. S.; Sikder, D.; Kodadek, T. *J. Comb. Chem.* **2007**, *9*, 592–600.

 10.1021/ja900669k CCC: $40.75

transcription up to 7-fold in vitro. Furthermore, one of the amphipathic isoxazolidine derivatives increased activity of a reporter gene in cells by a factor of 80, when tethered to the ligand-binding domain of a nuclear receptor.[15]

We previously developed a small molecule that binds to human Sur-2 coactivator, a ras-linked subunit of human mediator complex.[16] This wrench-shaped molecule inhibits expression of the Her2 oncogene by blocking interaction between the activation domain of ESX and its coactivator, Sur-2.[16,17] When covalently attached to a hairpin polyamide DNA-binding molecule, wrenchnolol is capable of activating transcription of a reporter gene in vitro. Biochemical evidence suggests that wrenchnolol activates transcription by recruiting human mediator complex and RNA polymerase II to the promoter through interaction with Sur-2.[18] The potency of wrenchnolol as an activation module in cultured mammalian cells was not previously examined.

Results of the current study show that wrenchnolol and its derivatives serve as activation modules in cells. Thirteen derivatives of wrenchnolol were chemically synthesized and evaluated in vitro and in cultured mammalian cells. One molecule exhibited potent induction of reporter genes, both in vitro and in cells, and generated biologically significant outputs in cells.

Results

Recruitment of Human Mediator Complex by Wrenchnolol. Naturally occurring activation domains recruit human mediator complex to promoters for gene activation. To determine if wrenchnolol is similarly capable of recruiting the mediator complex to a given site through interaction with Sur-2 coactivator, we performed a biotin−avidin pulldown experiment with a biotinylated wrenchnolol. The aminopentyl handle of wrenchnolol was selected as the site of biotinylation because previous NMR experiments showed that the handle stays away from the interface of the interaction.[16] Wrenchnolol was biotinylated through a methylene linker (Figure 1A), and the resulting conjugate (molecule **1a**) was incubated with HeLa nuclear extracts for 12 h at 4 °C. Bound proteins were recovered by avidin agarose resins and separated by SDS-PAGE. Western blot analysis of an SDS gel showed that molecule **1a** recruited Sur-2 protein and other components of human mediator complex (CDK8, TFIIE, and TFIIH), whereas control molecule **1b** exhibited no detectable recruitment (Figure 1B). Therefore, further experiments focused on molecule **1a**.

Transcriptional Activation by Molecule 1a in Vitro. To examine the ability of molecule **1a** to activate transcription, we conducted an in vitro assay, in which transcription of a reporter gene was directly monitored in the presence of HeLa nuclear extracts (Figure 2A). The reporter gene was a so-called G-less reporter, in which five binding sites of yeast Gal4 transcription factor control the production of G-less 130-base mRNA. A chimeric protein, consisting of the Gal4 DNA-binding domain and streptavidin (Gal4-SA), was preincubated with molecule

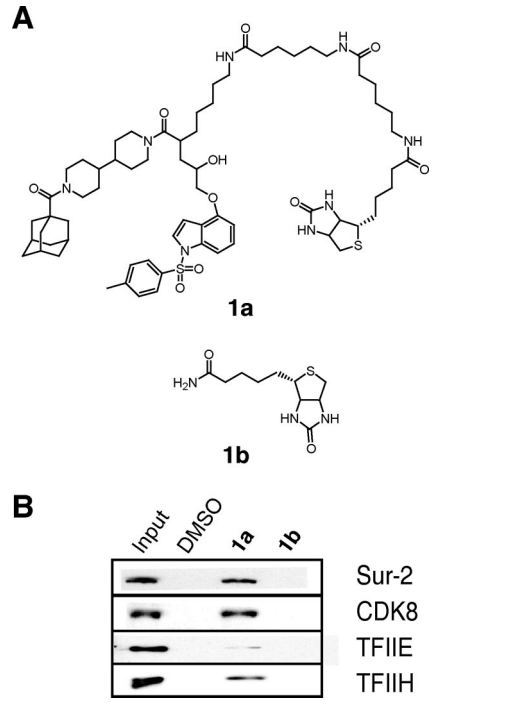


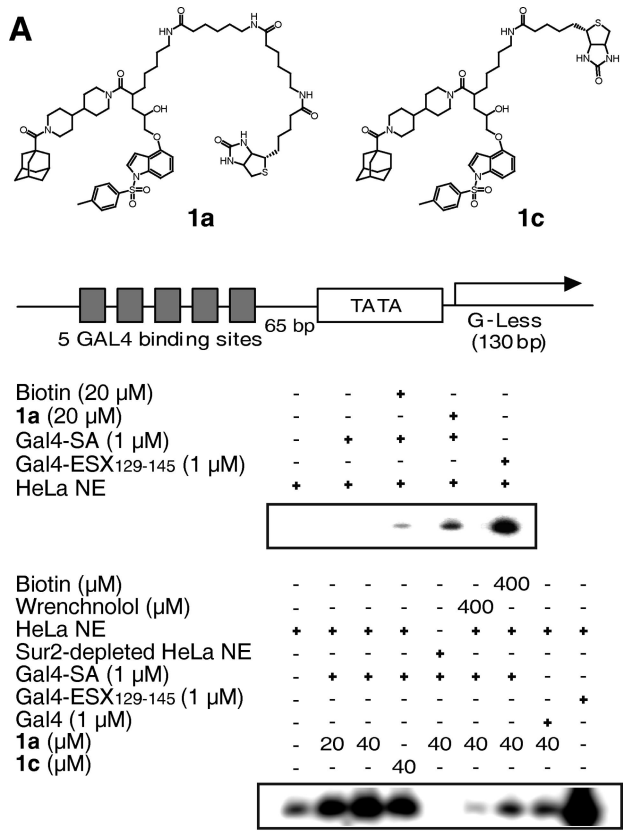
Figure 1. Recruitment of Sur-2 and other components of human mediator complex by biotinylated wrenchnolol (molecule **1a**). (A) Chemical structures of molecules **1a** and **1b**. (B) Molecule **1a** (5 μM) was incubated with 1 mg of HeLa nuclear extracts; bound proteins were purified by avidin resins and analyzed by Western blots with antibodies against Sur-2, Cyclin-dependent kinase 8 (CDK8), Transcription factor II E (TFIIE), and Transcription factor II H (TFIIH). Control experiments with DMSO alone or molecule **1b** are also shown.

1a, allowing Gal4-SA and molecule **1a** to interact via the biotin−streptavidin interaction. The transcription reaction was initiated by adding nuclear extracts and nucleotides, including ^{32}P-labeled CTP and 3′-O-methyl GTP. The reaction was allowed to proceed for 90 min at 37 °C, and mRNA products were separated on a denatured polyacrylamide gel.

Molecule **1a** activated transcription of the reporter gene in the presence of Gal4-SA (Figure 2A), although more weakly than the naturally occurring ESX activation domain fused with the Gal4 DNA-binding domain (Gal4-ESX$_{129-145}$). Molecule **1a** exhibited no detectable activation without Gal4-SA, and no activation occurred when excess biotin was added, indicating that gene activation by molecule **1a** is mediated by the streptavidin−biotin interaction. Addition of excess wrenchnolol or immunodepletion of Sur-2 from HeLa nuclear extracts also impaired the ability of molecule **1a** to activate transcription, emphasizing the importance of the wrenchnolol-Sur-2 interaction to activity of molecule **1a**. A wrenchnolol−biotin conjugate with a shorter linker (molecule **1c**) still activated transcription, but its activity was weaker than that of molecule **1a**, perhaps due to better projection of the wrenchnolol moiety in molecule **1a**. These results demonstrate that molecule **1a** activates transcription in vitro when tethered to the promoter of a reporter gene.

Wrenchnolol mimics the activation domain of ESX transcription factor; therefore, wrenchnolol and the ESX activation domain (ESX$_{129-145}$) should compete with each other for gene activation. Addition of excess amounts of wrenchnolol reduced the ability of Gal4-ESX$_{129-145}$ to activate expression of the reporter gene. Similarly, addition of ESX$_{129-145}$ decreased gene activation mediated by molecule **1a** (Figure 2B). These results support the hypothesis that the ESX activation domain and

(14) Minter, A. R.; Brennan, B. B.; Mapp, A. K. *J. Am. Chem. Soc.* **2004**, *126*, 10504–10505.
(15) Rowe, S. P.; Casey, R. J.; Brennan, B. B.; Buhrlage, S. J.; Mapp, A. K. *J. Am. Chem. Soc.* **2007**, *129*, 10654–10655.
(16) Shimogawa, H.; Kwon, Y.; Mao, Q.; Kawazoe, Y.; Choi, Y.; Asada, S.; Kigoshi, H.; Uesugi, M. *J. Am. Chem. Soc.* **2004**, *126*, 3461–3471.
(17) Asada, S.; Choi, Y.; Uesugi, M. *J. Am. Chem. Soc.* **2003**, *125*, 4992–4993.
(18) Kwon, Y.; Arndt, H. D.; Mao, Q.; Choi, Y.; Kawazoe, Y.; Dervan, P. B.; Uesugi, M. *J. Am. Chem. Soc.* **2004**, *126*, 15940–15941.



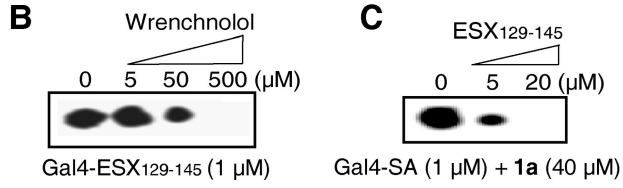


Figure 2. Transcriptional activation of a reporter gene by molecule **1a** in vitro. (A) A reporter gene in which expression of a 130-base G-less gene is controlled by five Gal4 sites was incubated with molecules **1a** or **1c** (20 or 40 μM) and HeLa nuclear extracts in the presence or absence of $Gal4_{1-94}$ fused with streptavidin (Gal4-SA) at 37 °C for 90 min. The ^{32}P-labeled 130-base G-less mRNA product was separated on a denatured polyacrylamide gel and visualized by autoradiography. Note that molecule **1a** failed to activate transcription when Sur-2 was depleted from nuclear extracts (Sur-2-depleted NE). For the competition assays, 400 μM wrenchnolol or biotin was added to each reaction mixture. (B) Transcriptional activation by 10 μM Gal4-$ESX_{129-145}$ was blocked by increasing concentrations of wrenchnolol (5−500 μM). (C) Transcriptional activation by molecule **1a** in the presence of Gal4-SA was blocked by increasing concentrations of $ESX_{129-145}$ (5−20 μM).

molecule **1a** activate transcription in a similar way, probably by binding to Sur-2.

Transcriptional Activation by Molecule 1a in Cells. The ability of molecule **1a** to activate transcription was also examined in cultured human cells. HeLa cells were cotransfected with a plasmid expressing Gal4-SA protein and a reporter gene in which five Gal4-binding sites control the expression of secreted alkaline phosphatase (SEAP). Seventeen hours after transfection, the cells were washed with phosphate-buffered saline and incubated for 24 h in serum-free medium containing biotinylated wrenchnolol (molecule **1a** or **1c**). Activation of the reporter gene was monitored by measuring the phosphatase activity of SEAP with a fluorogenic substrate (4-methylumbellyferyl phosphate). Treatment with molecule **1a** or **1c** increased expression of the SEAP reporter gene by a factor of 3.5 and 2.5, respectively (Figure 3A). The enhancement was suppressed when the expression vector of Sur-$2_{352-625}$, a wenchnolol-binding domain of Sur-2,[16] was cotransfected (Figure 3B). These results indicate that biotinylated wrenchnolol activates the reporter gene, although weakly, by binding to Sur-2 in cultured cells.

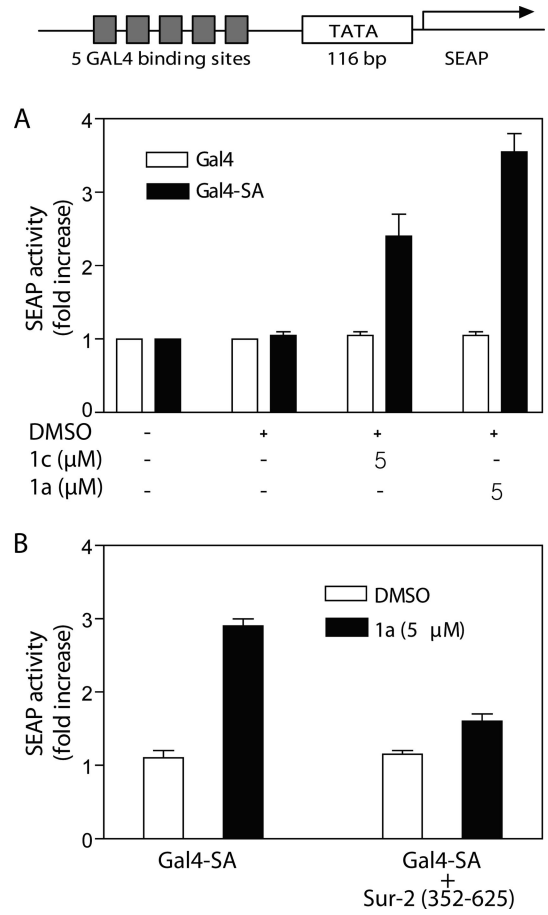


Figure 3. Transcriptional activation of a reporter gene by molecule **1a** in cells. (A) HeLa cells were cotransfected with an expression vector of Gal4-SA and a reporter gene in which expression of a secreted alkaline phosphatase (SEAP) gene is controlled by five Gal4 sites. Seventeen hours after the transfection, the cells were treated with molecule **1a** or **1c** (5 μM) in serum-free medium for 24 h. (B) Molecule **1a**-mediated transcriptional activation was blocked by overexpression of a wrenchnolol-binding domain of Sur-2 (Sur-$2_{352-625}$).

Directed Expression of an Endogenous Gene by Wrenchnolol. The ability of molecule **1a** to activate the expression of endogenous genes, rather than reporter genes, in cells was tested. Differentiation of C2C12 myoblasts into myotubes was chosen as a model. This morphological differentiation is induced by a single transcription factor, MyoD.[19] MyoD is a master regulator of myogenesis and activates the genes involved in differentiation to skeletal muscle cells.[20,21]

We first established a stable C2C12 cell line that expresses a chimeric protein of the DNA-binding domain of MyoD (amino

(19) Weintraub, H.; Tapscott, S. J.; Davis, R. L.; Thayer, M. J.; Adam, M. A.; Lassar, A. B.; Miller, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5434–5438.
(20) Rawls, A.; Morris, J. H.; Rudnicki, M.; Braun, T.; Arnold, H. H.; Klein, W. H.; Olson, E. N. *Dev. Biol.* **1995**, *172*, 37–50.
(21) Zhang, W.; Behringer, R. R.; Olson, E. N. *Genes Dev.* **1995**, *9*, 1388–1399.

acids 91−170) and streptavidin. This fusion protein, MyoD-SA, was expected to recruit molecule **1a** to the promoters of endogenous MyoD-responsive genes through the biotin−streptavidin interaction, enabling molecule **1a** to activate MyoD-responsive genes. When the cells were treated with molecule **1a** for 1 week, formation of long tubular myotubes was observed. In contrast, treatment with molecule **1b**, wrenchnolol, or DMSO caused little morphological alteration of the cells. One prominent characteristic of myotubes is the presence of multiple nuclei in a cell. To quantify the differentiation rates, we stained the nuclei of the cells and counted the number of cells having more than five nuclei in six microscopic fields. The percentage of differentiated cells was calculated on the basis of the total number of cells. Repeated experiments showed that molecule **1a** induced differentiation, in a dose-dependent manner, up to 12% at 1.25 μM (Figure 4B,C). Cells treated with molecule **1b** or DMSO exhibited about two nuclei, and differentiation rates were close to zero. Furthermore, molecule **1a** failed to induce differentiation of a control cell line that expresses the DNA-binding domain of MyoD only (data not shown).

We also examined the expression of myogenin, a representative marker of myogenesis induced by MyoD. Myogenin belongs to a family of MRF proteins, whose expression contributes to the final differentiation of the muscle cells.[22] Cells incubated with molecule **1a** expressed higher levels of myogenin than cells treated with control molecule **1b** or DMSO (Figure 4A). These results collectively suggest that wrenchnolol is capable of activating endogenous genes when tethered to specific promoters in cells.

Design and Synthesis of Wrenchnolol Derivatives. Having confirmed successful gene activation by molecule **1a** in our cell-based assays, our next step was to optimize wrenchnolol for such activity. Design of wrenchnolol derivatives was based on previous observations and the characteristics of naturally occurring activation domains.

The minimal activation peptide of ESX is rich in Ser residues, and one of the residues is located near the binding interface with Sur-2.[16,23] To mimic the side chain of the Ser residue, we designed a series of wrenchnolol derivatives (Figure 5, molecules **2a**−**c**) in which one or two hydroxy groups were introduced in the bipiperidine segment of wrenchnolol, a moiety that has been shown by NMR to be in close proximity to the binding interface. The bipiperidine segment of molecules **2a** and **2b** was synthesized from 4-(4′-piperidyl)pyridine: after partial reduction of the pyridine ring, a hydroxy group was introduced by hydroboration-oxidation (Scheme 1). Following acylation with adamantanecarbonyl chloride, the acetylated product was coupled with the other half of wrenchnolol to produce molecules **2a** and **2b**. To produce another wrenchnolol derivative, two hydroxy groups in **2c** were simultaneously generated by pinacol coupling of two 4-piperidone derivatives (Scheme 2). The resulting diol (molecule **2-22**) was conjugated with the other half of wrenchnolol, resulting in molecule **2c** (Scheme 2).

Other derivatives were produced by modifying a hydroxy group already present in the hydroxypropyl segment of wrenchnolol. The hydroxy group was acetylated (Figure 5, molecule **3a**) or switched to a carboxy group (molecule **3b**), based on the high occurrence of acidic amino acid residues in a number

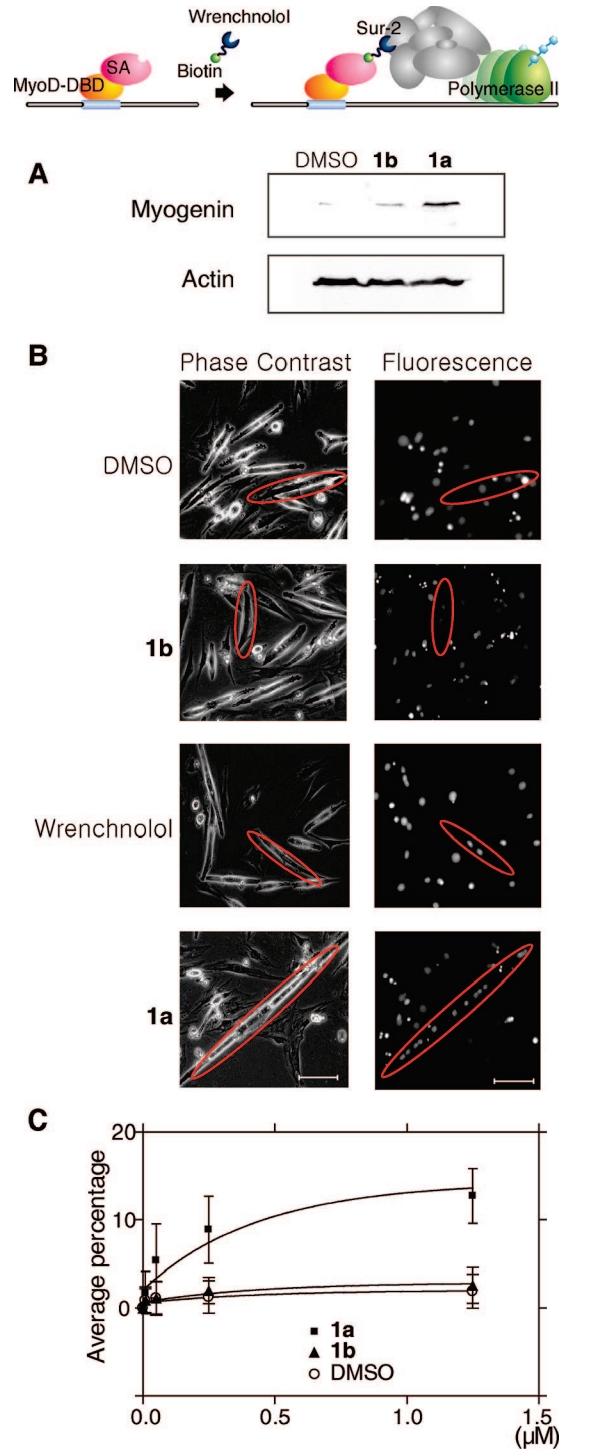


Figure 4. Induction of myogenesis by molecule **1a**. C2C12 myoblast cells were stably transfected with an expression vector encoding the DNA-binding domain of MyoD (MyoD-DBD) fused with streptavidin (SA). Myogenesis of the transfected cells was examined after treatment with molecule **1a**. (A) Western blot analysis of myogenin, a marker of myogenesis in the stably transfected C2C12 cells treated with molecule **1a** (1 μM) for 20 h. (B) Microscopic images of the stably transfected C2C12 cells after incubation with DMSO alone, molecule **1b** (1 μM), wrenchnolol (1 μM), or molecule **1a** (1 μM). Phase contrast and fluorescence images are shown after staining the nuclei with Hochest 33342. Red ovals indicate the size of a single cell. Scale bars indicate 20 μm. (C) Quantitative analysis of myotube formation. The numbers of cells with more than five nuclei were counted in six microscopic areas and expressed as a percentage of total cell number at each concentration of DMSO and molecules **1a** and **1b**. Error bars indicate ±SEM.

(22) Hollenberg, S. M.; Cheng, P. F.; Weintraub, H. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8028–8032.
(23) Asada, S.; Choi, Y.; Yamada, M.; Wang, S. C.; Hung, M. C.; Qin, J.; Uesugi, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12747–12752.

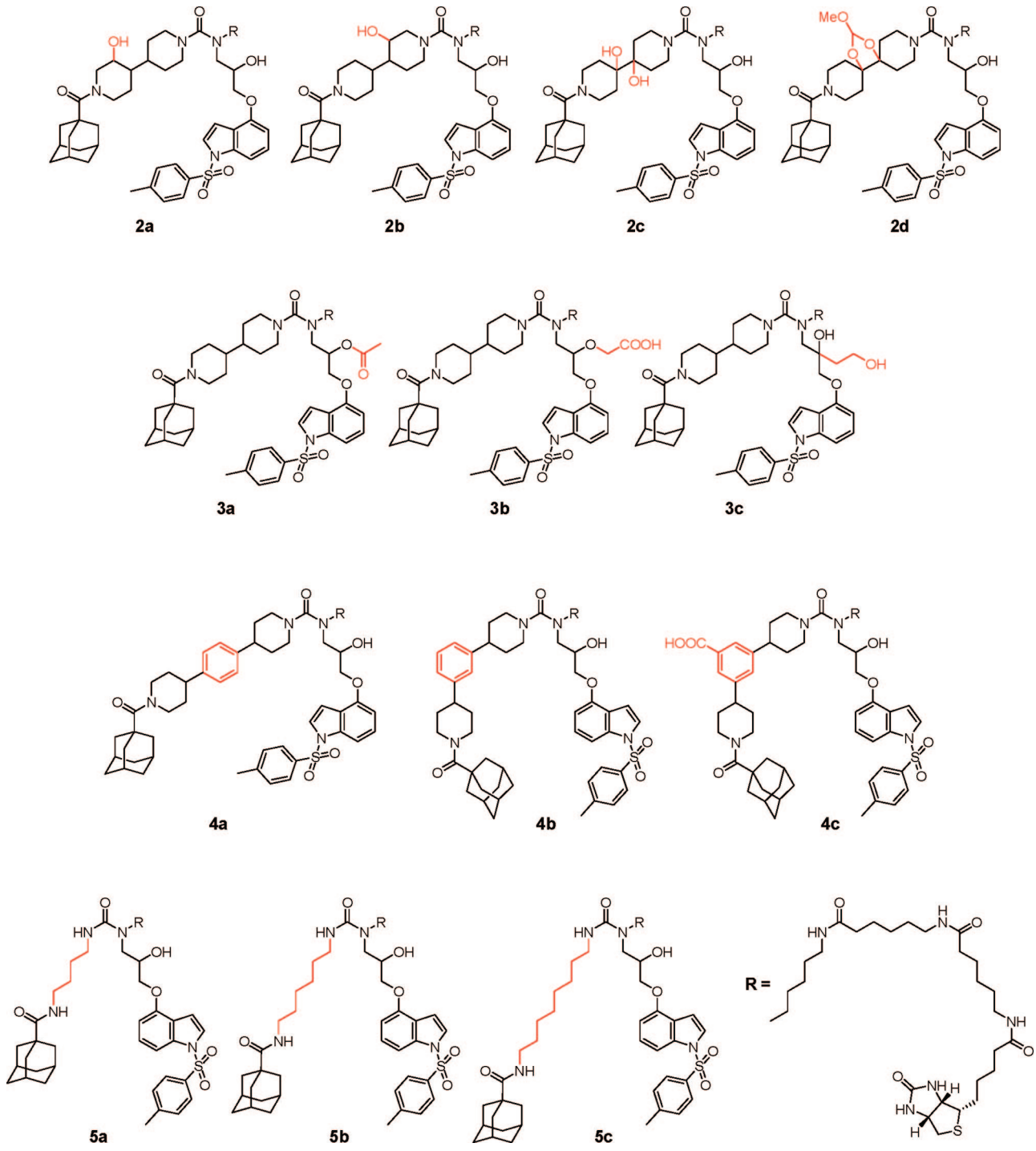


Figure 5. Chemical structures of wrenchnolol derivatives.

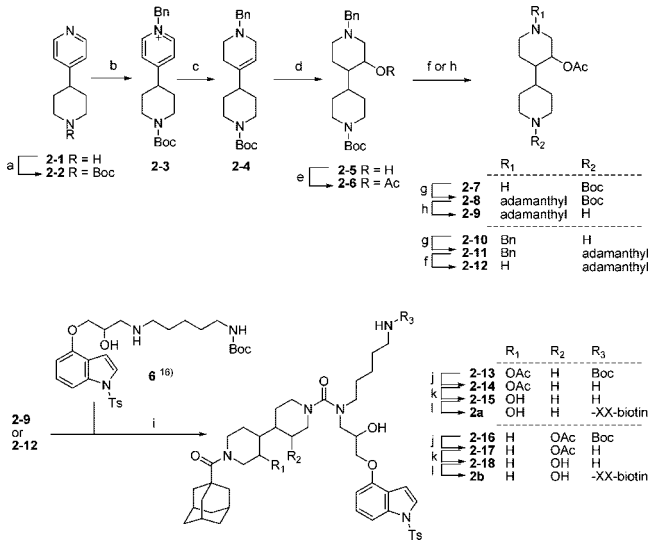
of potent activation domains.[24] In molecule **3c**, an extra hydroxy group was introduced near the existing hydroxy group: the two hydroxy groups were constructed using 3-chloro-2-chloromethyl-1-propene as a starting material for the indolyl half of the molecule (Scheme 3).

Results of previous NMR and synthetic studies suggested that the adamantyl and tosylated indolyl groups in wrenchnolol make direct contact with Sur-2.[16] To modulate the relative orientations of these two groups, a benzene ring was inserted into the bipiperidine segment of wrenchnolol at the para or meta position, producing molecules **4a** and **4b** (Figure 5). These molecules were synthesized from 4-phenylpiperidine (Scheme 4). After coupling with the adamantane segment, the second piperidine ring was introduced by Friedel−Crafts reaction to yield both para and meta products, molecules **4-3** and **4-4**, at a 7:10 ratio. Each purified product was coupled with the other half of the molecule.

In molecules **5a**−**c** (Figure 5), the bipiperidine segment of wrenchnolol was substituted with a series of methylene linkers, allowing flexibility in conformation. Such modifications were expected to generate interesting effects on gene activation because many naturally occurring activation domains have no

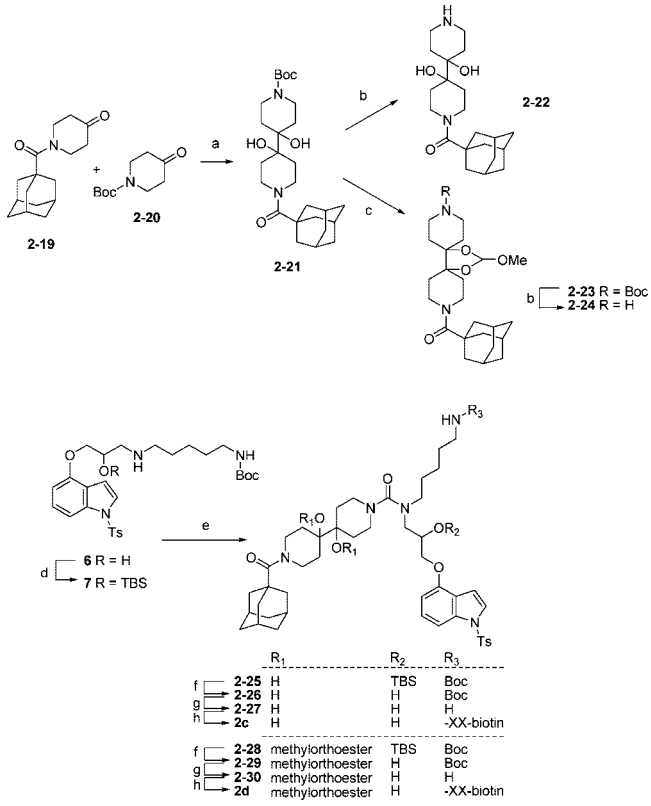
(24) Tjian, R.; Maniatis, T. *Cell* **1994**, *77*, 5–8.

Scheme 1[a]



[a] Conditions: (a) $(Boc)_2O$, $CHCl_3$, rt. (b) BnBr, CH_3CN, rt. (c) $NaBH_4$, CH_3OH, rt. (d) (1) BH_3 dimethylsulfide complex, THF, rt. (2) 30% H_2O_2, 1 N NaOH(aq), rt. (e) Ac_2O, pyridine, rt. (f) H_2, 10% $Pd(OH)_2$, CH_3OH, rt. (g) adamantanecarbonyl chloride, $CHCl_3$, rt. (h) TFA, $CHCl_3$, rt. (i) (1) CDI, THF, reflux (2) CH_3I, CH_3CN, rt. (3) amine **6**, Et_3N, CH_2Cl_2, rt. (j) TFA, $CHCl_3$, rt. (k) 1 N NaOH−MeOH, 45°C. (l) Biotin-XX-NHS, Et_3N,DMSO, rt.

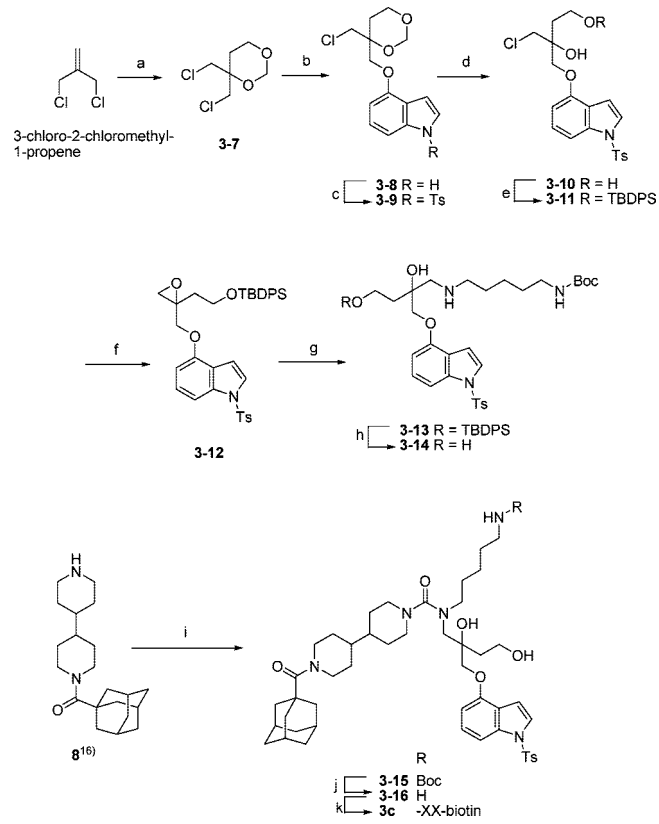
Scheme 2[a]



[a] Conditions: (a) Mg, HgCl, $TiCl_4$, THF, rt. (b) TFA, $CHCl_3$, rt. (c) Trimethyl orthoformate, benzoic acid, 150°C (d) TBSCl, Et_3N, THF, rt. (e) (1) CDI, THF, reflux (2) CH_3I, CH_3CN, rt. (3) Amine **2**-**22** or **2**-**24**, Et_3N, CH_2Cl_2, rt. (f) TBAF, CH_2Cl_2, rt. (g) TFA, $CHCl_3$, rt. (h) Biotin-XX-NHS, Et_3N,DMSO, rt.

preorganized structures and often bind multiple proteins in an induced-fit manner.[25]

Scheme 3[a]



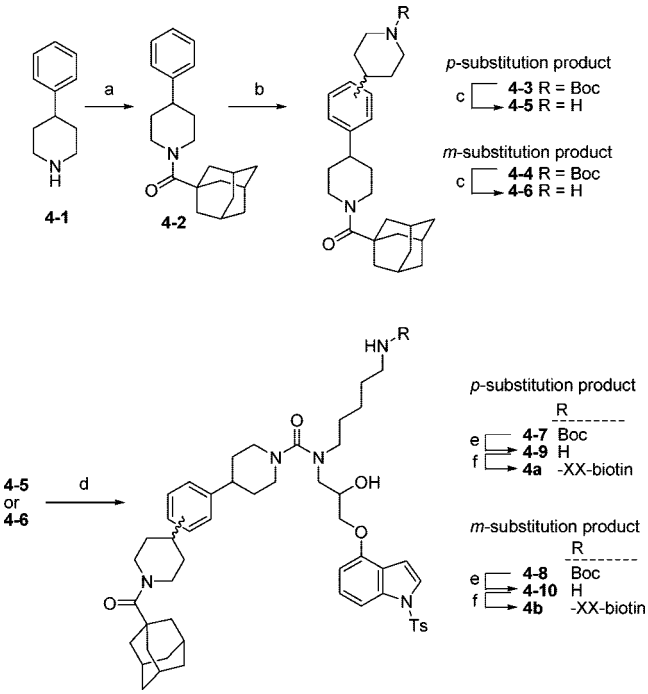
[a] Conditions: (a) Paraformaldehyde, H_2SO_4, CH_2Cl_2, rt. (b) 4-Hydroxyindole, KOH, DMF, 120 °C (c) NaH, TsCl, THF, 0 °C. (d) H_2SO_4, CH_3OH, 60 °C. (e) TBDPSCl, imidazole, DMF, rt. (f) NaH, THF, reflux. (g) *N*Boc 1,5-diaminopentane, CH_3OH, reflux. (h) TBAF, CH_2Cl_2, rt. (i) (1) CDI, THF, reflux. (2) CH_3I, CH_3CN, rt. (3) Amine **3**-**14**, Et_3N, CH_2Cl_2, rt. (j) TFA, $CHCl_3$, rt. (k) Biotin-XX-NHS, Et_3N,DMSO, rt.

Evaluation of the Wrenchnolol Derivatives for Gene Activation. The 13 derivatives of wrenchnolol were first tested for gene activation using in vitro transcription assays. Nine derivatives (Figure 6A, molecules **2a**, **2b**, **2c**, **3a**, **4a**, **4c, 5a**, **5b**, and **5c)** stimulated transcription of the G-less reporter gene as much as or more than molecule **1a**. Among those nine, molecules **2a**, **2c**, **3a**, **4a**, and **4c** had activity comparable to that of the ESX activation domain. Interestingly, introduction of a carboxy group or an extra hydroxy group in the hydroxypropyl segment (molecules **3b** and **3c**) abolished activity. In contrast, acetylation of the hydroxy group (molecule **3a**) increased gene activation, suggesting that a hydrophobic group in this position enhances the molecule's effectiveness as a gene activator. Modulation of the orientation between the adamantane and tosylated indole groups had drastic effects on activity. Para-insertion of a benzene group into a bipiperidine segment (molecule **4a**) increased activity, while meta-insertion (molecule **4b**) completely eliminated activity. Projection of hydroxy group(s) in the bipiperidine segment (molecules **2a** and **2c**) generally increased activity; this enhancement was canceled by protection of the hydroxy groups (molecule **2d**).

Wrenchnolol derivatives that were active in vitro were further evaluated in HeLa cells. Among the nine derivatives, molecules **2a** and **4a** exhibited enhanced activation of the Gal4-responsive reporter gene in comparison to molecule **1a**, and their activity was dose-dependent (Figure 6B). At a concentration of 5 μM,

(25) Triezenberg, S. J. *Curr. Opin. Genet. Dev.* **1995**, *5*, 190–196.

Scheme 4[a]



[a] Conditions: (a) Adamantanecarbonyl chloride, Et_3N, $CHCl_3$, rt. (b) (1) 4-Bromopiperidine, $AlCl_3$, 120 °C (2) $(Boc)_2O$, $CHCl_{23}$, rt. (c) TFA, $CHCl_3$, rt. (d) (1) CDI, THF, reflux (2) CH_3I, CH_3CN, rt. (3) Amine **6**, Et_3N, CH_2Cl_2, rt. (e) TFA, $CHCl_3$, rt. (f) Biotin-XX-NHS, Et_3N,DMSO, rt.

molecule **4a** exhibited 3−4 times greater activation of the reporter gene than molecule **1a**. All other derivatives, except molecules **2a** and **4a**, had limited activity in cells: molecules **2c**, **3a**, and **4c**, which were as potent as molecules **2a** and **4a** in vitro, failed to show comparable activity in cells.

Molecules **2a** and **4a**, the two derivatives active both in vitro and in cells, were tested for their ability to induce differentiation of C2C12 cells expressing MyoD-SA. Both molecules were more effective than molecule **1a** in promoting the formation of myotubes (Figure 6C). Molecule **4a** was more potent than molecule **2a** in inducing myogenesis, which is consistent with the results of the cell-based reporter gene experiments. At a concentration of 2 μM, molecules **2a** and **4a** induced differentiation into myotubes in 20% and 45% of the cell population, respectively (Figure 6D).

Biochemical Analysis of Molecule 4a. The high potency of molecule **4a** in vitro and in cells may reflect both molecule-specific cell permeability and the molecule's ability to recruit Sur-2. When molecules **1a**, **1b**, **2a**, and **4a** were incubated with HeLa nuclear extracts and avidin resins, and the recruited proteins were isolated and analyzed by Western blot analysis, molecule **4a** recruited the greatest amount of Sur-2, 14 times the amount recruited by molecule **1a** (Figure 6E).

The dissociation constants (K_D) of molecules **1a** and **4a** were compared using recombinant Sur-2. Fluorescein-labeled versions of the molecules (Figure S1, molecules **9** and **10**) were synthesized, and their fluorescence polarization was monitored in the presence of varying amounts of Sur-$2_{391-487}$, a Sur-2 fragment that binds to wrenchnolol. The K_D of molecule **10** was estimated to be 0.32 μM, while the K_D of molecule **9** was 0.84 μM, indicating that molecule **4a** binds 2.6 times more tightly to Sur-2 than molecule **1a**.

Discussion

Wrenchnolol is a small molecule that mimics the activation domain of transcription factor ESX. Our results indicate that wrenchnolol, similarly to the ESX activation domain, activates gene expression by interacting with Sur-2 and recruiting the human mediator complex. However, wrenchnolol exhibited low potency, only 3.5-fold activation in cultured cells, which was lower than activation by naturally occurring domains.

One possible reason for the weak activation exhibited by wrenchnolol may be our use of the biotin−streptavidin interaction for bridging a DNA-binder and an activator. The biotin−streptavidin interaction is nontoxic, has excellent selectivity, is unrelated to transcriptional regulation, and is inert in activating transcription. However, a number of biotin-binding proteins are abundant in mammalian cells, and these proteins may squelch the activity of biotinylated molecules, reducing the sensitivity of the assays.

Despite wrenchnolol's low potency, we observed the induction of reporter and endogenous genes by biotinylated wrenchnolol (molecule **1a**) in cultured human cells. The detection of myogenesis provided a particularly reliable, sensitive, and biologically relevant assay that permitted the optimization of wrenchnolol for gene activation. MyoD is known to have a positive autoregulatory loop in cells: this master regulator induces its own gene.[26] Perhaps weak induction of the MyoD gene through MyoD-responsive elements in the promoter may have driven an activation cascade of MyoD and other myogenic genes. The stable expression of MyoD-SA in cells may also account for the success of this assay in detecting wrenchnolol-induced gene activation. MyoD-SA has no activation domain but binds to MyoD-responsive elements in the promoter, making it a decoy, or inhibitor, of endogenous MyoD. In fact, parental, untransfected C2C12 cells differentiated more spontaneously to myotubes under the condition we used than the cells transfected with an expression vector of MyoD-SA. Biotinylated wrenchnolol had no detectable effects on this spontaneous differentiation while it increased the differentiation of the MyoD-SA cells. Conversion of the inhibitor to an activator by addition of biotinylated wrenchnolol may have contributed to the detectable effects on myogenesis.

Comparison of the wrenchnolol derivatives indicated that molecules with extra hydroxy groups, but not carboxy groups, tend to be active both in vitro and in cells. A hydroxy group was similarly found to be essential for the transcriptional activation of an amphipathic isoxazolidine molecule.[14,15] Although the target protein of the isoxazolidine molecule may be different from that of the wrenchnolol derivatives, molecules with hydroxy groups may be generally effective in activating transcription.

The most potent derivative synthesized in this study was molecule **4a**, in which a benzene ring was inserted into the bipiperidine segment of wrenchnolol at the para position. Interestingly, the meta-substituted analogue (**4b**) was completely inactive, suggesting that orientation of the adamantane segment of wrenchnolol has profound effects on activity. On the basis of their different K_D values, the greater activity of molecule **4a** is due in part to stronger binding to Sur-2. Nevertheless, the lower K_D value of molecule **4a** may not fully explain its increased ability to activate transcription and recruit Sur-2 protein from nuclear extracts. Another possible explanation is

(26) Thayer, M. J.; Tapscott, S. J.; Davis, R. L.; Wright, W. E.; Lassar, A. B.; Weintraub, H. *Cell* **1989**, *58*, 241–248.

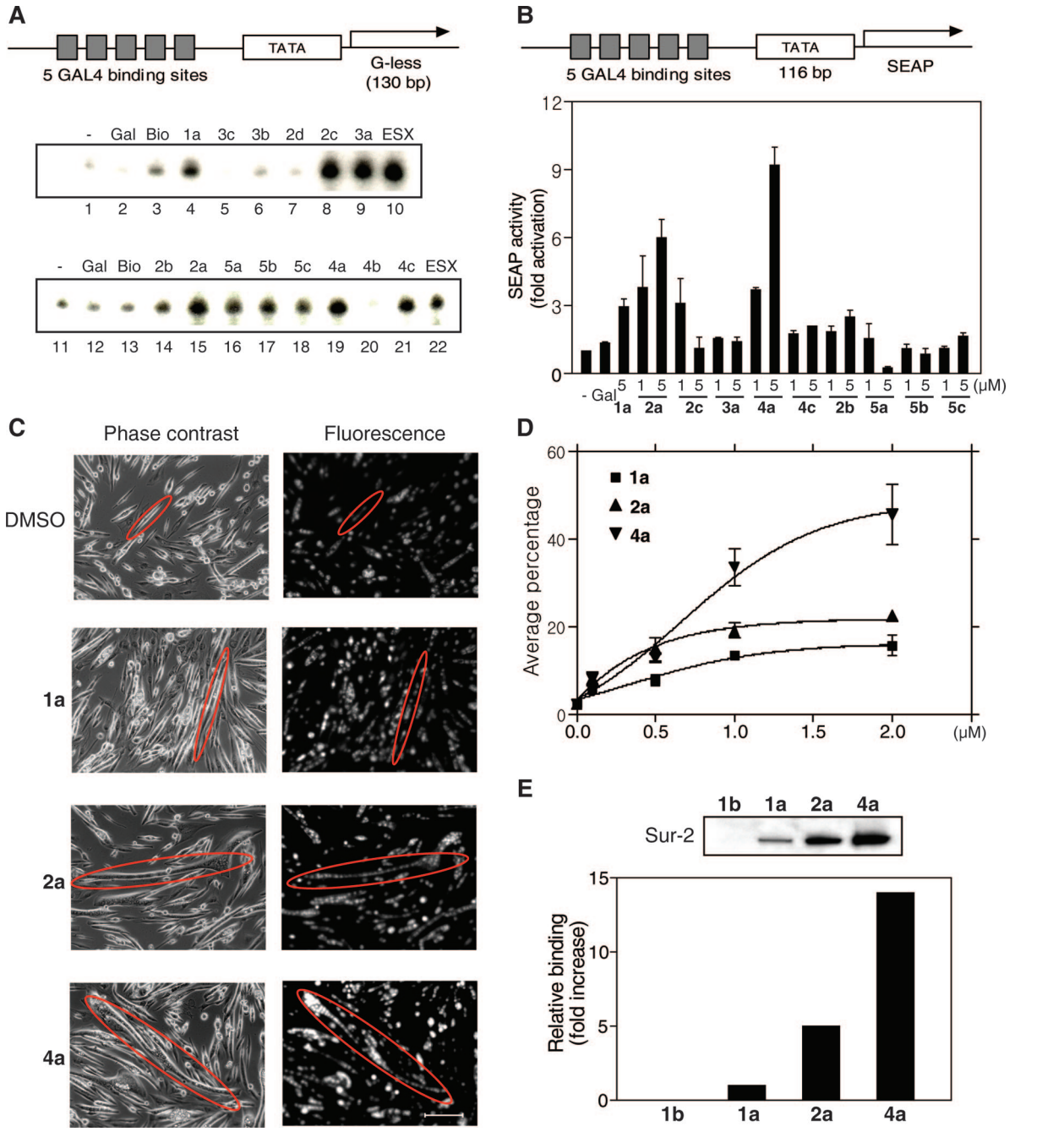


Figure 6. Evaluation of wrenchnolol derivatives in vitro and in cells. (A) In vitro assays. The derivatives were tested for their ability to activate transcription of a reporter gene, in which expression of a 130-bp G-less gene is controlled by five Gal4-binding sites. The transcriptional activation was evaluated by detecting radio-labeled mRNA transcripts on a denatured polyacrylamide gel. (B) Cell-based assays. Molecules **2a**, **2c**, **3a**, **4a**, **4c**, **2b**, **5a**, **5b**, and **5c** were evaluated for their activity in cells. HeLa cells were transfected with a Gal4-responsive secreted alkaline phosphatase (SEAP) reporter gene and an expression vector of a Gal4−streptavidin fusion protein. The transfected cells were incubated with 1 or 5 μM of one of the wrenchnolol derivatives for 24 h. Expression levels of SEAP were evaluated by monitoring the fluorescence changes of a fluorogenic substrate (4-methylumbellyferyl phosphate). (C) Myogenesis assays with molecules **2a** and **4a**. C2C12 myoblast cells were stably transfected with an expression vector encoding the DNA-binding domain of MyoD fused with streptavidin. The stably transfected cells were treated with 1 μM of molecule **2a** or **4a** for 7 days. The nuclei of the cells were stained with Hochest 33342; phase contrast and fluorescence images are shown. Red ovals indicate the size of a single cell. Scale bars indicate 20 μm. (D) Quantitative analysis of myotube formation. The numbers of cells with more than five nuclei were counted in six microscopic areas and expressed as a percentage of total cell number at each concentration of the molecules. Error bars indicate ±SEM. (E) Analysis of Sur-2 recruitment. Molecule **1a**, **2a**, or **4a** (5 μM) was incubated with 1 mg of HeLa nuclear extracts for 12 h at 4 °C. Bound proteins were purified using avidin beads and analyzed by Western blot analysis (top). Quantitative analysis of the bands was performed with Image J (bottom).

that molecule **4a** may bind other coactivator proteins in addition to Sur-2 to recruit the mediator complex, a characteristic often observed in naturally occurring activation domains. Alternatively, molecule **4a** may be more selective for Sur-2 than wrenchnolol. If the activity of wrenchnolol is squelched in cells by additional binding proteins that are irrelevant to transcriptional regulation, slight improvement in selectivity to Sur-2 would significantly enhance activity by increasing the availability of the molecule. Greater understanding and further optimization of the enhanced activity of molecule **4a** could lead

to the design of small molecule transcriptional regulators with broad applications.

Selective activation or inhibition of gene expression is essential to understanding complex cellular events. Selective down-regulation of gene expression by siRNA has been widely accepted as a tool for biological studies. Although a new RNA-based technology for gene activation is currently emerging,[27] targeted up-regulation of genes has been more challenging. Results of the present study showed that wrenchnolol activates gene expression in cells when it is tethered to a DNA-binding molecule. Stronger gene activation was achieved by the optimized wrenchnolol derivative, molecule **4a**, generating biologically significant outputs in cells. This optimized derivative may serve as a starting point for designing biological tools or components of fully synthetic transcription factors that permit selective up-regulation of genes.

Experimental Section

Synthesis of Wrenchnolol Derivatives. A complete description of experimental procedures and compound characterization data is provided in the Supporting Information.

In Vitro Transcription Assay. The reporter plasmid used was pTATA-G5, in which the transcription of a 130-base G-less gene is controlled by five Gal4-binding sites. Each biotinylated wrenchnolol derivative was incubated with HeLa nuclear extracts,[28] 150 ng of a G-less reporter plasmid, and a Gal4-DNA-binding domain (amino acids 1−94) fused with streptavidin (Gal4-SA; final 1 μM). The in vitro transcription assays are described in detail in our previous work.[18] Briefly, transcription was initiated by adding ^{32}P-labeled CTP and unlabeled nucleotides (600 μM ATP, 600 μM UTP, 300 μM 3′-O-methyl GTP, 6 μM CTP) to the reaction mixture. Each reaction tube was incubated at 37 °C for 90 min. Gal4-fusion with the ESX activation domain (amino acids 129−145) was used as a positive control (Gal4-ESX, final concentration = 1 μM). The transcribed RNA was purified by phenol extraction, precipitated in ethanol, and separated using a denatured 6% polyacrylamide gel. The 130-base mRNA product of transcription was monitored by autoradiography. For the competition assays, increasing amounts of wrenchnolol (5−500 μM) or $ESX_{129-145}$ (5−20 μM) were added to the reactions. $ESX_{129-145}$ was synthesized by standard solid phase synthesis as described.[23] Sur-2-depleted HeLa nuclear extracts were prepared by immunodepletion of endogenous Sur-2 protein from the nuclear extracts, as previously described.[18]

Reporter Gene Assays in Cells. The reporter gene used was pSEAP (Clontech), in which the transcription of a secreted alkaline phosphatase (SEAP) gene is controlled by five tandem copies of a representative Gal4-binding sequence, 5′-GTCCTCCGAGCGGA-3′. The reporter gene (0.5 μg) was cotransfected into HeLa cells on 24-well plates, with an expression plasmid encoding streptavidin fused with a Gal4 DNA-binding domain (amino acids 1−94). Seventeen hours after the transfection, the medium was changed with a serum-free DMEM containing 1 or 5 μM of wrenchnolol derivative, and incubated further for 24 h. SEAP activity was measured as described.[29]

C2C12 Differentiation Assay. C2C12 myoblasts were purchased from the ATCC and maintained according to ATCC instructions. The cells were stably transfected with an expression vector of the MyoD DNA-binding domain (amino acids 91−170), fused with streptavidin using FuGENE6 (Roche). The stable cell lines were selected with 0.5 mg/mL of Geneticin (Invitrogen). The stably transfected cells were spread onto a six-well plate, and allowed to reach 100% confluence in a DMEM medium containing 20% fetal bovine serum. The medium was changed to a DMEM medium containing 10% horse serum and each molecule at 0−2 μM. The medium was replaced with fresh medium containing each chemical every 24 h for 7 days. The nuclei of the cells were stained with Hochest 33342, and differentiated cells with more than five nuclei were counted in six microscopic fields.

In Vitro Recruitment Assay. HeLa nuclear extracts (1 mg) were incubated with 5 μM biotinylated wrenchnolol or one of its derivatives in a 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1% (v/v) NP-40 for 12 h at 4 °C. Bound proteins were precipitated with immobilized neutravidin beads (PIERCE). The samples were washed three times with the same buffer; bound proteins were eluted from the beads by boiling in SDS-PAGE sample buffer and separated by SDS-PAGE. An antibody against Sur-2 (Santa Cruz Biotechnology) was used for Western blot analyses.

Fluorescence Polarization Assay. A GST fusion of a Sur-2 protein fragment, GST-$Sur\text{-}2_{391-487}$, was bacterially expressed, purified by using glutathione−sepharose resins, and used for affinity measurements. The affinity of the fluorescein-labeled wrenchnolol derivatives was measured by monitoring changes in fluorescence polarization in the presence of varying concentrations of GST-$Sur\text{-}2_{391-487}$. GST-$Sur\text{-}2_{391-487}$ was serially diluted from 30.4 to 0.48 μM in phosphate buffered saline (pH 7.4) containing a fluorescein-labeled wrenchnolol derivative (0.5 μM). The samples were allowed to equilibrate, and binding was measured by fluorescence polarization (excitation at λ 490 nm, emission at λ 519 nm) on a Perkin LS55 spectrophotofluorometer. Data were analyzed using IGOR Pro software (WaveMetrics. Inc.), and the K_D values were obtained by curve fitting.

Acknowledgement. This work was supported by PRESTO/JST to M.U. The research group of M.U. participates in the global COE program "Integrated Material Science" (#B-09). S.K. is a postdoctoral fellow of JSPS. We thank the Hamachi research group for access to their fluorescence spectrometer and the members of the Uesugi research group for helpful discussion and support.

Supporting Information Available: Details of the synthetic procedures and NMR data, results of fluorescence polarization experiments, and complete ref 11. This material is available free of charge via the Internet at http://pubs.acs.org.

JA900669K

(27) Janowski, B. A.; Younger, S. T.; Hardy, D. B.; Ram, R.; Huffman, K. E.; Corey, D. R. *Nat. Chem. Biol.* **2007**, *3*, 166–173.
(28) Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. *Nucleic Acids Res.* **1983**, *11*, 1475–1489.
(29) Choi, Y.; Asada, S.; Uesugi, M. *J. Biol. Chem.* **2000**, *275*, 15912–15916.