A Synthetic Library of Cell-Permeable Molecules

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Abstract: Small molecules that induce or stabilize the association of macromolecules have proven to be useful effectors of a wide variety of biological processes. To date, all examples of such chemical inducers of dimerization have involved known ligands to well-characterized proteins. The generality of this approach could be broadened by enabling the discovery of heterodimerizers that target known macromolecules having no established ligand, or heterodimerizers that produce a novel biologic response in screens having no predetermined macromolecular target. Toward this end, we report the construction of a diversified library of synthetic heterodimerizers consisting of an invariant ligand that targets the FK506-binding protein (AP1867) attached to 320 substituted tetrahydrooxazepines (THOXs). The THOX components were generated by a combination of liquid- and solid-phase procedures employing sequential Mitsonobu displacements to join two structurally diversified olefin-containing monomers, followed by ruthenium-mediated olefin metathesis to effect closure of the seven-membered ring. The 320 resin-bound THOX ligands were coupled in parallel to AP1867, and the products were released from the resin to yield candidate heterodimerizers in sufficient yield and purity to be used directly in biologic testing. A representative panel of 25 candidate heterodimerizers were tested for their ability to pass through the membrane of human fibrosarcoma cells, and all were found to possess activity in this tissue culture system. These studies pave the way for further studies aimed at using small-molecule inducers of heterodimerization to effect novel biological responses in intact cells.

Introduction

Small molecules that induce or stabilize the association of macromolecules hold tremendous promise as biological effectors. Such "chemical inducers of dimerization" (CIDs) are well known in Nature.[1,2] For example, the anti-cancer agents adriamycin[3] and etoposide[4] act by stabilizing a catalytic intermediate having topoisomerase II bound to its nicked DNA substrate. The immunosuppressive agent FK506 induces the dimerization of FK506-binding protein (FKBP) and calcineurin (Cn), thereby abrogating the latter's protein phosphatase activity (Figure 1A).[5,6] The immunosuppressive agent cyclosporin A (CsA) also blocks Cn activity by an induced dimerization mechanism, but in this case the partner protein is cyclophilin.[5,7] Yet a third small-molecule immunosuppressant, rapamycin, induces the association of FKBP to the FK506/rapamycin associated protein (FRAP).[8−10]

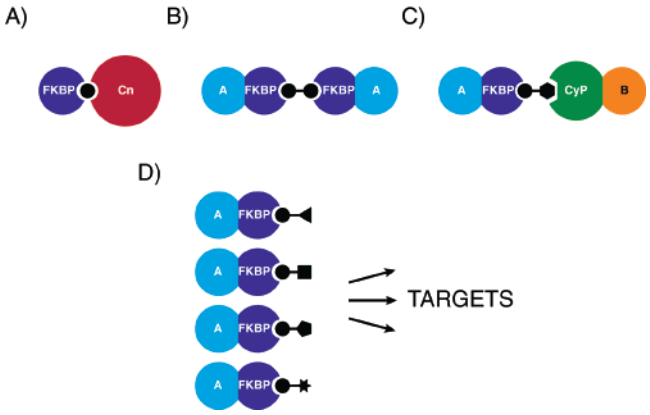

Figure 1. Small molecules can induce the dimerization of macromolecules. (A) The macrolide natural product FK506 induces the dimerization of two proteins, the FK506-binding protein (FKBP) and calcineurin (Cn). (B) Synthetically linked dimers of FK506 induce the homodimerization of a protein having FKBP fused to a partner A. (C) Synthetically linked dimers of FK506 and cyclosporin A (CsA) induce the heterodimerization of two fusion proteins, one of which has an FKBP domain and the other of which has a cyclophilin (CyP) domain. (D) Heterodimerizers having the FK506 analogue AP1867 linked to a diversity element can be used to induce the dimerization of a fusion protein with diverse intracellular targets. A and B represent proteins to which FKBP and CyP are fused in a contiguous polypeptide chain by ligation of the corresponding coding DNA sequences. A and B typically possess a function that is useful in a biologic context, such as DNA binding, transcriptional activation, fluorescence, nuclear export or import, or intracellular signaling.

Recognizing the potential of designed synthetic CIDs to bring together signaling proteins in a spatially and temporally

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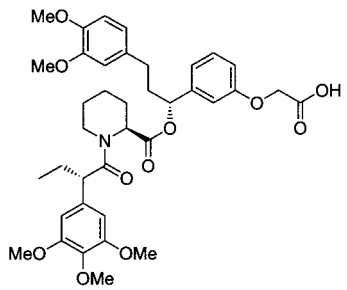
controlled manner, Schreiber and Crabtree have demonstrated the ability to engineer a variety of inducible signal transduction processes into cells (Figure 1B,C).[11−15] Numerous variations on the general theme of using induced dimerization to generate biological responses have been reported, thus underscoring the broad scope of this strategy.[16−34]

The foregoing examples of synthetic "dimerizers" all involve bifunctional molecules having two ligand moieties linked together, each of which binds a known receptor. An exciting, and as-yet unexplored possibility is to induce the association of one known macromolecule with an unknown one, thereby creating biological relationships that Nature may not have explored. Furthermore, dimerizing two known macromolecules can present a challenge when a ligand is known for one partner but not the other. Both these lines of research could be pursued by the construction of bifunctional small-molecule libraries having an invariant ligand attached to a diversified ligand whose structure varies among the members of the library (Figure 1D). Here we report the construction of the first such diversified library of synthetic candidate heterodimerizers.

Results and Discussion

Invariant Receptor−Ligand Pair. The invariant ligand of our candidate heterodimerizers should bind with high affinity and specificity to an intracellular receptor, without disrupting a function that is essential for cell survival. Preferentially, the ligand structure should bias toward cell permeability and be synthetically accessible. To satisfy these criteria, we chose AP1867, a synthetic analogue of the natural products FK506 and rapamycin.[19]



AP1867

AP1867 associates with wild-type FKBP (K_d = 67 nM) but has a much higher affinity (K_d = 94 pM) for an engineered mutant of FKBP having a mutation of Phe 36 to Val (FKBP*).[19] This difference in affinity for FKBP* vs FKBP makes it possible to target AP1867 to the engineered protein selectively, even in the presence of high concentrations of the wild-type endogenous FKBP. A synthetic homodimer of AP1867 readily permeates mammalian cells, thus increasing the likelihood that heterodimerizers containing this moiety would also be cell-permeable.[19]

Design of the Core Structure of the Diversified Ligand. Our design strategy considered various aspects of receptor−ligand interactions, cell physiology, and feasibility of solid-phase synthesis to provide final products having sufficient purity. Taking these issues into account, we selected the tetrahydrooxazepine (THOX) core shown in Scheme 1. This semirigid core presents three diversity elements, two on the ring and one on a side chain. The three stereogenic centers on the THOX unit can be independently varied via asymmetric synthesis, thus giving rise to all eight possible stereoisomers.

The presence of uncharged heteroatoms on the THOX unit at physiological pH balances the hydrophobicity of the molecule, thus favoring solubility in aqueous media and transit across cell membranes. From a synthetic standpoint, the THOX unit provides independent attachment points to both the solid support and the invariant ligand, and the modular structure of THOX suggests a convergent synthesis. Finally, we were intrigued by the possibility that the N−O bond of THOX might be reductively cleaved to convert the semirigid cyclic library to a more flexible acyclic library containing additional diversity sites.[35]

We envisioned producing the THOX system (Scheme 1) by coupling two independently diversifiable monomers through Mitsunobu chemistry,[36] followed by ring-closing olefin metathesis (RCM)[37,38] to produce exclusively the heterocoupled product having cis double bond geometry. The chemical sensitivity of AP1867 suggested it would be prudent to attach

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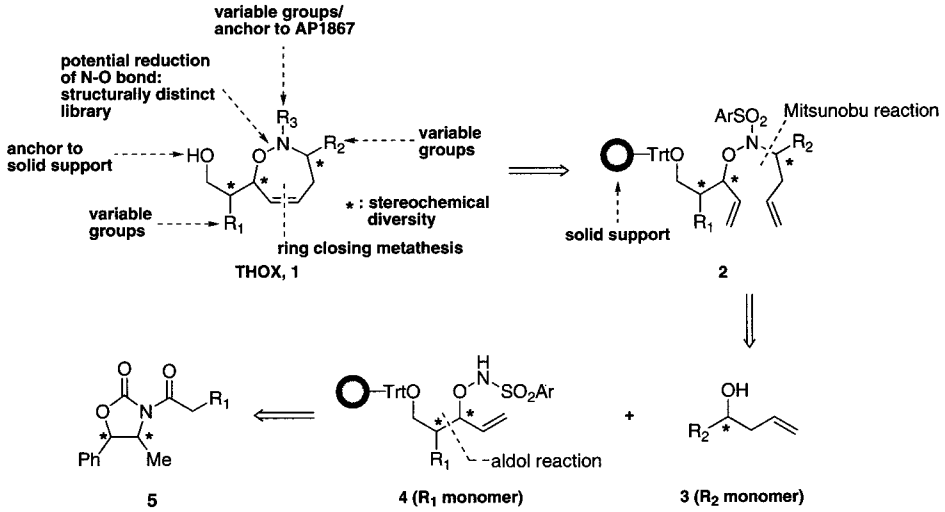
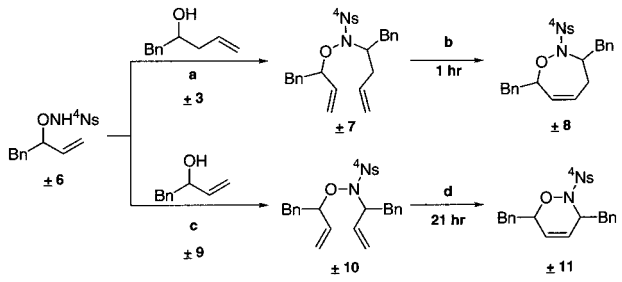
Scheme 1



Scheme 2[a]



[a] Reagents and conditions: (a) $EtO_2C-N=N-CO_2Et$, Ph_3P, THF, 0 → 23 °C, 1 h, 66%; (b) 0.05 equiv of $Cl_2Ru(PCy_3)_2=CHPh$, CH_2Cl_2 (0.02 M), 1 h, 23 °C; 55% isolated yield at 100% conversion; (c) $EtO_2C-N=N-CO_2Et$, Ph_3P, THF, 0 → 23 °C, 1 h, quantitative; (d) 2 × 0.05 equiv of $Cl_2Ru(PCy_3)_2=CHPh$, CH_2Cl_2 (0.1 M), 23 °C, 21 h; 45% isolated yield at 100% conversion.

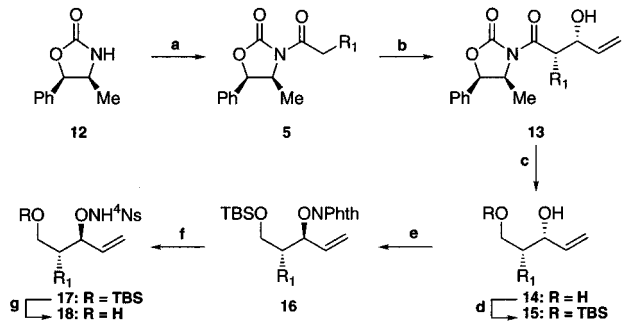
this moiety to THOX in the penultimate synthetic step, leaving only cleavage of the complete heterodimerizer from the solid support, an operation that can be carried out under mild conditions.

Model Study for the RCM Reaction Step. The most critical step of our synthetic scheme is RCM to form the cis endocyclic olefin. To our knowledge, no literature precedent exists for olefin metathesis on substituents containing an N−O bond in the newly formed ring; hence, we were uncertain as to whether the relative rates of the RCM step would be significantly influenced by the relative stereochemistry at the sp^3 centers. To address these issues experimentally, we coupled racemic sulfonylalkoxyamine **6** to racemic homoallylic alcohol **3** and subjected the entire mixture of stereoisomers to RCM (Scheme 2). With 5 mol % catalyst, cyclization was complete within 1 h at room temperature. The 1:1 mixture of diastereomers obtained revealed the lack of a significant stereochemical influence on the rate of RCM. These results indicate that the THOX ring is formed rapidly enough for combinatorial applications, and furthermore is capable of producing all possible stereoisomers.

We also examined RCM on the coupling product of **6** and racemic allylic alcohol **9**, leading to the corresponding product containing a six-membered ring. Although this RCM process also produced a 1:1 mixture of diastereomers, the reaction was sluggish, requiring 21 h to achieve completion. We therefore chose the THOX system for initial library development.

Asymmetric Synthesis of Monomers. To avoid the need for the deconvolution of complex stereochemical mixtures during library screening, we decided to employ only stereochemically defined substrates in the library synthesis. To set the relative and absolute stereochemistry of the R_1 monomer, we selected the Evans chiral auxiliary-directed aldol reaction (Scheme 3).[39,40] Known oxazolidinone **12** was deprotonated with *n*-butyllithium and then coupled with commercially available acid chlorides to generate imides **5** in quantitative yield. Alternatively, the oxazolidinone auxiliary was coupled to carboxylic acids using 2-chloro-1-methylpyridinium iodide or dicyclohexylcarbodiimide (DCC)/triethylamine/catalytic 4-(dimethylamino)pyridine, in 79−94% yield.

Scheme 3[a]



[a] Reagents and conditions: (a) *n*-BuLi in hexanes, THF; R_1CH_2COCl, THF, −78 → 23 °C, 30 min, quantitative; or $R_1CH_2CO_2H$, 2-chloro-1-methylpyridinium iodide or DCC, DMAP, Et_3N, 0 → 23 °C, then 23 °C, 1.5 h, 79−94%; (b) **5**, *n*-Bu_2BOTf, $^iPr_2NEt$, 0 °C, 20 min; acrolein, CH_2Cl_2, −78 → 0 °C, 40 min, 55−79%; (c) $LiBH_4$, Et_2O, 0 °C, 5 min, 81−90%; (d) TBSCl, Et_3N, CH_2Cl_2, 0 → 23 °C, 8 h, 80−89%; (e) PhthNOH, Ph_3P, $EtO_2C-N=N-CO_2Et$, 0 → 23 °C, 45 min, 67−71%; (f) $MeNH_2$, EtOH, CH_2Cl_2, 23 °C, 15 min; 4-nitrobenzenesulfonyl chloride, 2,4,6-collidine, CH_2Cl_2, 0 °C, 15 min, 98% (crude); (g) HF−pyridine, THF, 0 °C, then 23 °C, 10 h, 65−73%. DMAP, 4-(dimethylamino)pyridine; TBS, Si^tBuMe_2; Phth, phthalimide; ^{4}Ns, 4-nitrobenzenesulfonyl.

The imides were then condensed with acrolein under standard conditions to furnish β-hydroxyl imides **13** in 55−79% yield. In all cases, the main diastereomer accounted for greater than 95% of the isolated product following column chromatography. 1,3-Diols **14**, prepared by chemoselective reduction of imides **13**, were regioselectively protected as the *tert*-butyldimethylsilyl (TBS) ethers to afford secondary alcohols **15**.

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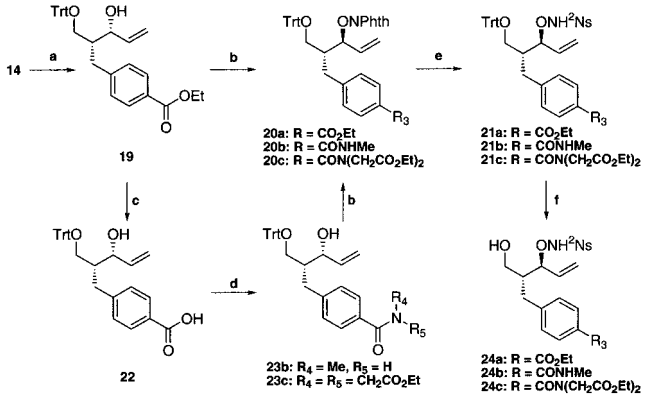
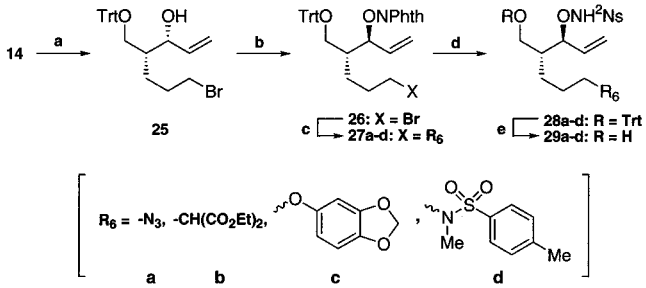
Scheme 4[a]



[a] Reagents and conditions: (a) TrtCl, pyridine, CH_2Cl_2, 0 °C, 2 h, 91%; (b) PhthNOH, Ph_3P, $EtO_2C{-}N{=}N{-}CO_2Et$, 0 → 23 °C, 45 min, 72−97%; (c) LiOH, H_2O_2, MeOH/THF/H_2O (2:3:1), 46 °C, 7 h, 98%; (d) **22**, 2-chloro-1-methylpyridinium iodide, $^iPr_2NEt$, CH_2Cl_2, 0 °C, then 23 °C, 15 min; $NH(CH_2CO_2Et)$ or $MeNH_2$, 0 °C, then 23 °C, 15 min, 58% to quantitative; (e) $MeNH_2$, EtOH, CH_2Cl_2, 23 °C, 15 min; 2-nitrobenzenesulfonyl chloride, 2,4,6-collidine, CH_2Cl_2, 0 °C, 15 min, 71−83% and 98% (crude); (f) formic acid/Et_2O (1:1), ethanethiol or mercaptoacetic acid, 23 °C, 2 h, 29−75%. Trt, trityl; ^{2}Ns, 2-nitrobenzenesulfonyl.

Scheme 5[a]



[a] Reagents and conditions: (a) TrtCl, pyridine, CH_2Cl_2, 0 °C, 2 h, 88%; (b) PhthNOH, Ph_3P, $EtO_2C{-}N{=}N{-}CO_2Et$, 0 → 23 °C, 45 min, 74%; (c) nucleophile, base, DMF, 50% to quantitative; (d) $MeNH_2$, EtOH, CH_2Cl_2, 23 °C, 15 min; 2-nitrobenzenesulfonyl chloride, 2,4,6-collidine, CH_2Cl_2, 0 °C, 15 min, 26−93%; (e) formic acid/Et_2O (1:1), mercaptoacetic acid, 23 °C, 2 h, 42−82%.

The secondary alcohols were then converted to *N*-alkoxyphthalimides **16** under Mitsunobu conditions in 67−71% yield.[41,42] The phthalimides were converted to the corresponding 4-nitrobenzenesulfonyl derivatives **17** by a one-pot procedure (methylamine, followed by 4-nitrobenezenesulfonyl chloride and collidine) to generate a nucleophilic alkoxyamine. Treatment of the TBS ethers with HF−pyridine gave primary alcohols **18**.

R_1 monomers bearing functionalized side chains were synthesized by a slightly altered sequence (Schemes 4 and 5). For certain applications,[16] it would be desirable to have carboxylic acid or amide functionality in the diversified ligand. The synthesis of such monomers diverted from the common scheme at the 1,3-diol stage by protection of the primary hydroxyl as a trityl ether **19**, saponification of the aryl ester, and amide formation to give benzamides **23b**,**c**. The trityl-protected compounds were subjected to Mitsunobu displacement by *N*-hydroxyphthalimide, and the resulting products were converted by the aforementioned one-pot procedure to the sulfonylalkoxyamines **21a**−**c**. Deprotection of the trityl group was accomplished by treatment of sulfonylalkoxyamines **21a**−**c** with formic acid in diethyl ether to give primary alcohols **24a**−**c**.

A late-diversification scheme was used to generate additional monomers. Trityl-protected bromide **25** was chemoselectively converted to the corresponding *N*-alkoxyphthalimide **26** in 74% yield (Scheme 5).[43] Despite the electrophilicity of the *N*-alkoxyphthalimide moiety, we found it feasible to carry out efficient displacement of the bromide with a variety of nucleophiles in the presence of the phthalimide. The displacement products **27a**−**d** were then carried forward to the corresponding primary alcohols **29a**−**d**.

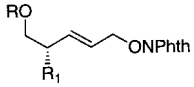
For both unfunctionalized and functionalized R_1 monomers, the overall yields based on the starting carboxylic acids or acid chlorides were 5−19% (Figure 2). The entire reaction sequence could be carried out in parallel on four monomers on a 10−50 g scale.

Synthesis of the R_2 Monomers. Just as with the R_1 monomers, the R_2 monomer series was designed to incorporate a variety of aliphatic, aromatic, and masked acidic functional groups (Figure 3). The 16 R_2 monomers were prepared enantioselectively using the Brown procedure for asymmetric allylation of aldehydes.[44] Bromobenzene derivatives were further elaborated by the Heck reaction to furnish cinnamate derivatives **3g** and **3h** (Scheme 6).[45,46] Methyl benzoate **3e** was converted to amide **3f** via hydrolysis, followed by standard amide formation.

Library Synthesis. With these building blocks in hand, we undertook the parallel synthesis of a THOX library, aiming to produce 6 μmol of each compound. To track the efficiency of the library synthesis, we randomly chose 40 out of the total 320 library members[47] and analyzed the entire course of the synthesis by HPLC and LC−MS analysis of a small portion of the resin removed after each reaction step.

The 20 R_1 monomers were condensed with a 38−75-μm polystyrene-based trityl chloride resin[48,49] in dichloromethane and pyridine to form trityl ethers **4** (Scheme 7).[50] Prior to library synthesis, resin-bound azide **29a** (Figure 2) was converted to

(41) The byproduct in this step resulted from S_N2' attack to yield the following compound:



(42) *N*-Hydroxy 4-nitrobenzenesulfonamide failed to react with the alcohol under Mitsunobu conditions.

(43) The alkyl bromide with one less carbon underwent undesired cyclopropane formation under mildy basic conditions and was unsuitable for R_1 monomer synthesis: ^{1}H NMR (400 MHz, $CDCl_3$, 23 °C) δ = 7.44−7.30 (m, 5H, Ar), 5.68 (d, 1H, J = 7.0 Hz, −C*H*Ph), 4.76 (dq, 1H, J = 7.0, 6.6 Hz, −NC*H*), 3.18 (m, 1H, −C*H*C═O), 1.16−1.13 and 1.04−1.00 (m, 4H, $-CH_2CH_2-$), 0.90 (d, 3H, J = 6.6 Hz, $-NCHCH_3$); CI^+ HRMS calcd for $C_{14}H_{19}N_2O_3$ ($M + NH_4^+$) 263.1396, found 263.1406.

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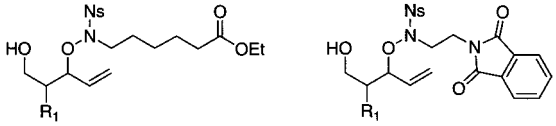
(47) In addition to the 320 THOX library members reported here, 40 additional compounds lacking the THOX core were synthesized by reacting the 20 R_1 monomers with two commercially available alcohols, ethyl 6-hydroxyhexanoate and *N*-(2-hydroxyethyl)phthalimide. Since these monomers have no olefin, they were not subjected to RCM, and are therefore open-chain alkoxyamines:



(48) Chen, C. X.; Randall, L. A. A.; Miller, R. B.; Jones, A. D.; Kurth, M. J. *Tetrahedron* **1997**, *53*, 6595−6609.

(49) Chen, C. X.; Randall, L. A. A.; Miller, R. B.; Jones, A. D.; Kurth, M. J. *J. Am. Chem. Soc.* **1994**, *116*, 2661−2662.

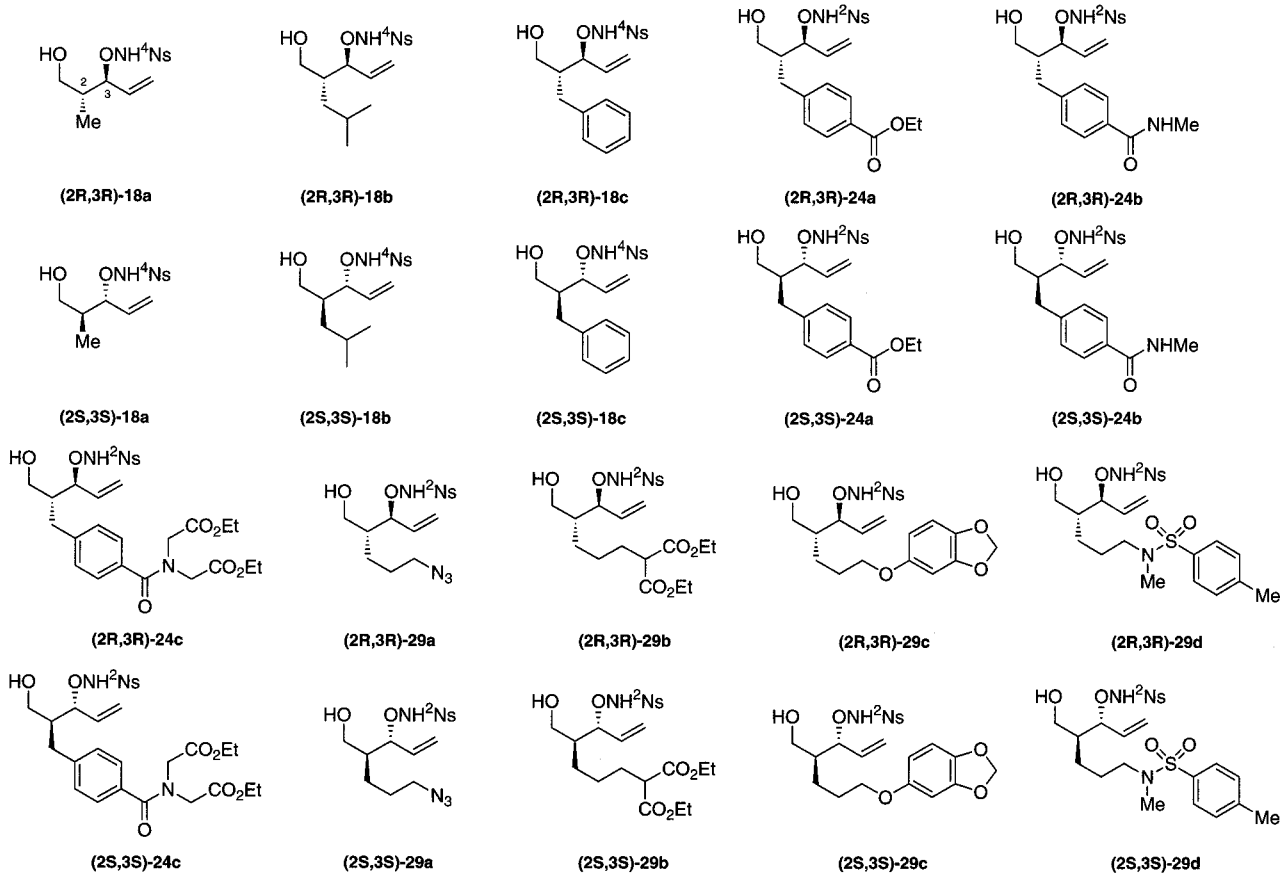


Figure 2. Structures of R_1 monomers.

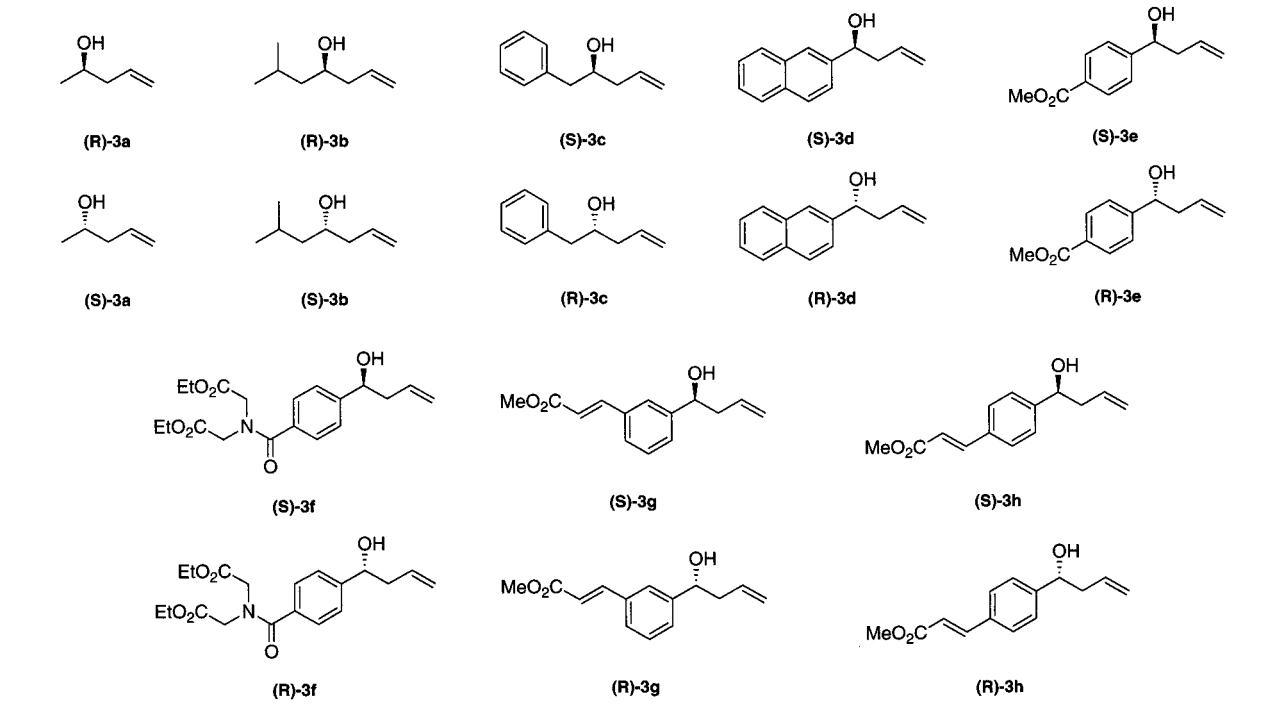


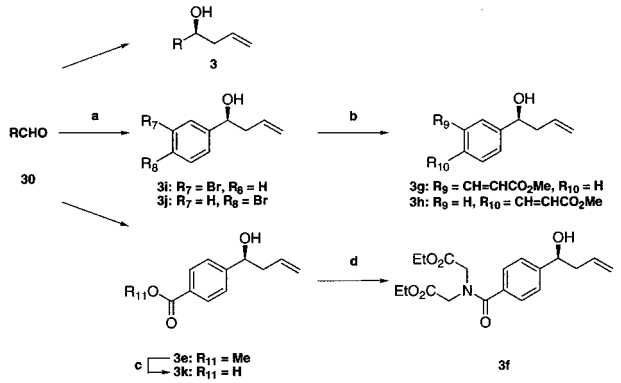
Figure 3. Structures of R_2 monomers.

the corresponding acetamide by treatment with neat thiolacetic acid ($RN_3 \rightarrow RNHAc$).[51] The 16 homoallyl alcohols (Figure 3) were activated by treatment with di-*tert*-butyl azodicarboxylate or diisopropyl azodicarboxylate and triphenylphosphine and then coupled to the alkoxysulfonamide nitrogen of the resin-bound R_1 monomers under Mitsunobu conditions, thereby producing 320 dienes **2**.[52] For all substrates examined, the RCM reaction was found to produce a single product with complete conversion.

(50) We experienced lower isolated chemical yields after intramolecular cyclization on the resin at high loading level (approximately 0.8 mmol/g). Although the loading capacity of the commercially available trityl chloride resin is greater than 0.8 mmol/g, we adjusted the stoichiometry so that the loading level of the R_1 monomers was at 0.2 mmol/g and the rest of trityl chloride was reacted with ethanol or methanol in the presence of triethylamine.

(51) Peters, S.; Bielfeldt, T.; Meldal, M.; Bock, K.; Paulsen, H. *Tetrahedron Lett.* **1992**, *33*, 6445−6448.

Scheme 6[a]



[a] Reagents and conditions: (a) (−)-*B*-methoxydiisopinocamphenylborane, allylmagnesium bromide, Et_2O, 0 → 23 °C, 1 h, then **30**, −78 → 23 °C, 3 h; NaOH, MeOH, and 30% H_2O_2, 23 °C, 72 h, 58−73%; (b) 0.035 equiv of $Pd(OAc)_2$, 0.13 equiv of $(o\text{-Tol})_3P$, 10 equiv of methyl acrylate, Et_3N, NMP, 90 → 110 °C, 4.5 h, 43%; (c), (d) see Scheme 4 (c), (d).

Of the 6 μmol of the resin-bound THOX library, 5 μmol was set aside at this stage for further library development, and 1 μmol was carried forward to generate the heterodimerizer library.

The deprotection of the nitrobenzenesulfonyl groups is noteworthy: under the standard conditions [thiophenol and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 1-methyl-2-pyrrolidinone (NMP)], the 2-nitrobenzenesulfonyl group was removed without any detectable side products.[53,54] In contrast, treatment of resin-bound library members having the 4-nitrobenzenesulfonyl group with the same reagents gave mostly side products, which arose by attack of thiophenol at aryl carbons other than the desired sulfamido-bearing carbon.[55]

We speculated that it might be possible to cleave the 4-nitrobenzenesulfonyl amide bond intramolecularly by including an additional nucleophilic center in the alkanethiol reagent. After screening several alkanethiols, we found that ethylene 1,2-dithiol or β-mercaptoethanol and DBU in methylene chloride gave the desired secondary alkoxyamine as the sole reaction product. Although we observed Michael addition products upon treatment of the cinnamate derivatives (THOX compounds containing R_2 monomers **3g** and **3h**) with thiol and DBU in NMP, subsequent β-elimination induced by DBU in NMP regenerated the original conjugated olefin cleanly.

In the next step, the secondary alkoxyamines were condensed with Fmoc-6-aminohexanoic acid in the presence of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine to generate Fmoc-protected amines **33**.[56] The presence of an aromatic ring adjacent to the carbon bearing the nitrogen in the seven-membered ring was found to retard the coupling reaction significantly. Increasing the concentration of the activated acid 3-fold and allowing the reaction to proceed for 18 h circumvented this problem. Under these modified conditions, the condensation was driven to completion.

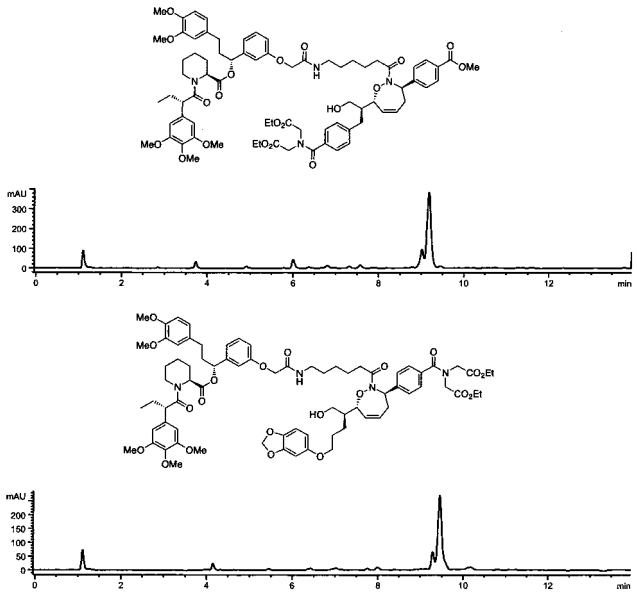
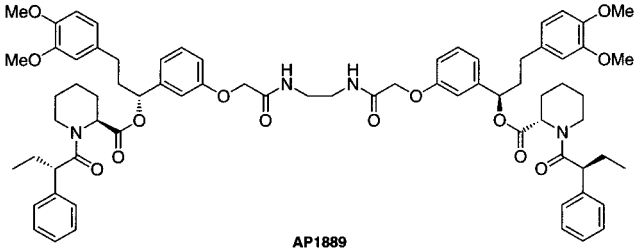


Figure 4. HPLC analysis of the final library compounds. Solid-phase reactions were monitored on a Hewlett-Packard 1100 HPLC, outfitted with an Eclipse XDB C_8 column (4.6 mm × 150 mm, 5 μm). Samples were eluted using a gradient of 35 → 100% acetonitrile in water containing 0.1% formic acid over 13 min (1.5 mL/min), and the UV absorbance was monitored at 220 (shown), 240, and 254 nm.

Deprotection of the Fmoc group under standard conditions (20% piperidine in DMF) liberated primary amines **34**. The amines were then coupled with AP1867 in the presence of HATU and diisopropylethylamine to provide resin-bound heterodimeric compounds **35**. Treatment of the resin with 1% trifluoroacetic acid (TFA) and 5% triisopropylsilane in methylene chloride for 1 min at room temperature released the 320 compounds into solution. Reverse-phase HPLC analysis of the final products revealed the purity of the crude products as released from the resin to be in the range of 60−80% (Figure 4). This level of purity satisfied our criteria for direct biological testing of the crude synthetic heterodimerizers.

Cell Permeability of the Candidate Heterodimerizers. For synthetic heterodimerizers to be useful in biological assays with intracellular targets, they must be cell-permeable. To assess the cell permeability of these compounds, we employed a published procedure.[17,18,20] This assay uses cells that express two FKBP* fusion proteins: one containing the ZFHD1 DNA-binding domain and the other containing the p65 acidic transcriptional activator domain (Figure 5). The small-molecule homodimerizer AP1889 brings these two protein components together, thereby activating transcription of the downstream encoding secreted alkaline phosphatase (SEAP). Upon activation, the SEAP is

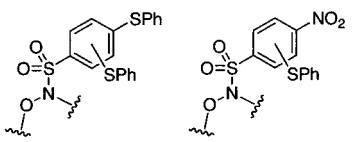


(52) Use of diethyl azodicarboxylate led to ethylation of the nitrogen to the extent of 1−3%; the ethyl donor is presumably ethanol, generated by partial decomposition of the diethyl azodicarboxylate.

(53) Wuts, P. G. M.; Northuis, J. M. *Tetrahedron Lett.* **1998**, *39*, 3889−3890.

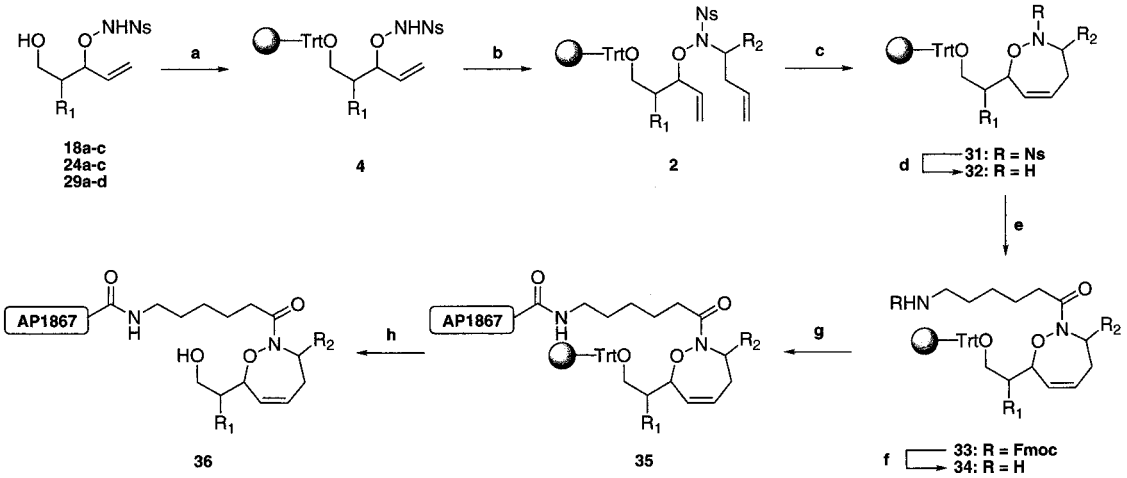
(54) Fukuyama, T.; Jow, C.-K.; Cheung, M. *Tetrahedron Lett.* **1995**, *36*, 6373−6374.

(55) ^{1}H NMR and LC−MS analysis indicates that the side products may have the following structures:



(56) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678−9683.

Scheme 7[a]



[a] Reagents: (a) Trt-Cl resin, pyridine, DCM then methanol or ethanol, pyridine, CH_2Cl_2; (b) R_2 monomers **3**, Ph_3P, $^iPrO_2C{-}N{=}N{-}CO_2{}^iPr$ or $^tBuO_2C{-}N{=}N{-}CO_2{}^tBu$, THF; (c) $Cl_2Ru(PCy_3)_2{=}CHPh$, CH_2Cl_2; (d) RSH, DBU, CH_2Cl_2 or NMP; (e) Fmoc-6-aminohexanoic acid, HATU, $^iPr_2NEt$, NMP; (f) piperidine, DMF; (g) AP1867, HATU, $^iPr_2NEt$, NMP; (h) TFA, $^iPr_3SiH$, CH_2Cl_2. Fmoc, 9-Fluorenylmethoxycarbonyl.

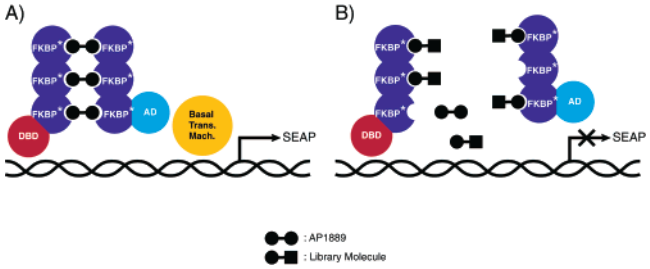


Figure 5. Assay for the cell permeability of synthetic heterodimerizers. (A) A synthetic homodimerizer, AP1889, induces the dimerization of two mutant FKBP domains (FKBP*), one linked to a DNA binding domain (DBD) and the other linked to a transcriptional activation domain (AD). The induced dimerization leads to recruitment of the basal transcriptional machinery and activation of the secreted alkaline phosphatase (SEAP) gene. The amount of SEAP secreted into the growth medium was determined as described (not shown).[11] (B) A cell-permeable synthetic heterodimerizer containing only one FKBP*-targeting ligand competing with the homodimerizer for FKBP*, leading to suppression of SEAP activation.

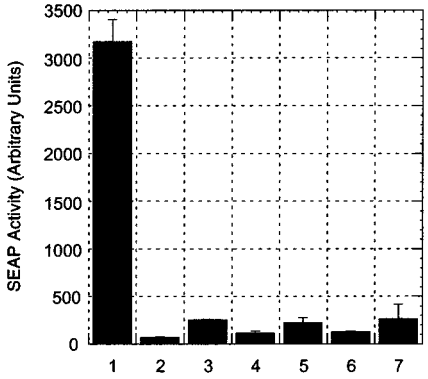


Figure 6. Permeability of synthetic heterodimerizers to human fibrosarcoma cells. The vertical axis corresponds to SEAP activity in arbitrary units. Lane 1: Induced activation of the SEAP gene by the homodimerizer AP1889 at a concentration of 10 nM. Lanes 2−7: Suppression by six different members of the heterodimerizer library at 100 nM in the presence of 10 nM AP1889. All assays were run in triplicate.

secreted by the cells into the medium, where its presence can be measured quantitatively using a fluorogenic substrate.

If a library molecule passes into the cell, it will compete with AP1889, thereby disrupting the complex that activates SEAP expression. Thus, loss of SEAP expression indicates that the library member is cell-permeable. A randomly chosen subset of the library comprising 25 heterodimerizers was screened separately at 500 nM concentration in the presence of 10 nM AP1889. At these high concentrations, each of the 25 compounds strongly inhibited SEAP expression. None of the tested compounds exhibited significant cytotoxicity, as determined by visual inspection of the adherent cells. Next, six of the most polar library members were screened in triplicate at a concentration of 100 nM in the presence of 10 nM AP1889, and again each gave strong inhibition of SEAP expression (Figure 6). These data provide a clear indication that the THOX-based heterodimerizers are cell-permeable and thus suitable for use in biological screens.

Conclusion

Here we have described the first example of a library constructed for the purpose of inducing the association of two proteins, only one of which has a known small-molecule ligand. No hard and fast rules exist for selecting the variant ligand in such a system, and in principle there exist a number of scaffolds that would serve this purpose. We elected to use the THOX system for its structural novelty in the cyclic form, its convertibility to a linear form through reduction of the N−O bond, and the opportunity it presents to use stereochemistry as a diversity element. Furthermore, the presence of the olefin in the THOX ligands should enable further functionalization, for example through reduction, epoxidation, or dihydroxylation. In the present work, we have described variation of both the stereochemistry and the attached functionality at two points in the THOX system. Straightforward elaborations of the general strategy outlined here should permit variation of the linkage between the variant and invariant ligand, with respect to length, functionality, and attachment position. This would have the beneficial effect of systematically altering the composite surface presented by the variant component and the protein to which it is bound (here, FKBP*).

An important criterion for any variant ligand in a heterodimerization system is that it must have a tendency to produce cell-permeable compounds when fused to the invariant ligand. The issue of cell permeability has seldom been analyzed systematically for components of combinatorial libraries. Here

we have analyzed a sample of the candidate heterodimerizers, representing a broad range of hydrophobicities, and found all of the tested components to be freely permeable to fibrosarcoma cells at a concentration of 100 nM. What remains to be demonstrated is whether any of the 320 library members reported in this study will function as heterodimerizers. Efforts along these lines are ongoing in a variety of biological assay systems. Several promising "hits" have already been identified in an assay that reports the ability of the molecules to induce nuclear export (J.M.F. and G.L.V., unpublished results).

Experimental Section

General Techniques. All reactions were carried out under an argon atmosphere with dry, freshly distilled solvents under anhydrous conditions, unless otherwise noted. Tetrahydrofuran (THF) and diethyl ether (Et_2O) were distilled from sodium−benzophenone, and methylene chloride (CH_2Cl_2) was distilled from calcium hydride. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise stated.

All reactions were monitored by thin-layer chromatography carried out on 0.25-mm E. Merck silica gel plates (60F-254) using UV light, 2.4% phosphomolybdic acid/1.4% phosphoric acid/5% sulfuric acid in water, or 0.2% ninhydrin in ethanol and heat as developing agents. TSI silica gel (230−400 mesh) was used for flash column chromatography. Preparative thin-layer chromatography (PTLC) separations were carried out on 0.50-mm E. Merck silica gel plates (60F-254).

Optical rotations were measured on a 241 polarimeter (Perkin-Elmer), and infrared spectra were taken with a 5ZDX FT-IR spectrophotometer (Nicolet). NMR spectra were recorded on Inova500, Inova500B, Mercury400, or Mercury400B (Varian) or AM300 (Bruker) instruments and calibrated using a solvent peak or tetramethylsilane as an internal reference. The following abbreviations are used to indicate the multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. High-resolution mass spectra were obtained by using an SX-102A mass spectrometer (JEOL) using a mass resolution of 5000 for CI^+ and 10 000 for ES^+/ES^-.

Polystyrene-based trityl chloride resin was obtained from NovaBiochem (200−400 mesh). Solid-phase reactions were monitored on a Hewlett-Packard 1100 HPLC, outfitted with an Eclipse XDB C_8 column (4.6 mm × 150 mm, 5 μm). Samples were eluted using a gradient of 35 → 100% acetonitrile in water containing 0.1% formic acid over 13 min (1.5 mL/min), and the UV absorbance was monitored at 220, 240, and 254 nm.

LC−MS spectra were obtained using a Micromass Platform LCZ mass spectrometer interfaced with a Waters 2690 Alliance HPLC, outfitted with a Waters Symmetry C_{18} column (2.1 mm × 50 mm, 3.5 μm). Samples were eluted using a gradient of 15 → 100% acetonitrile in water containing 0.1% formic acid over 10 min, and the UV absorbance, positive APCI, and negative APCI were collected.

Abbreviations. Ac, acetyl; Bn, benzyl; Bu, butyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMF, dimethylforamide; Et, ethyl; Et_2O, ethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; Fmoc, 9-fluorenylmethoxycarbonyl; Me, methyl; MeOH, methanol; NMP, 1-methyl-2-pyrrolidinone; ^{2}Ns, 2-nitrobenzenesulfonyl; ^{4}Ns, 4-nitrobenzenesulfonyl; Ph, phenyl; Phth, phthaloyl; TBS, *tert*-butyldimethylsilyl; TFA, trifluoroacetic acid; Tf, trifluoromethanesulfonyl; THF, tetrahydrofuran; Trt, triphenylmethyl.

(A) Preparation of Nitrobenzenesulfonyl Alkoxyamides 18a−c, 24a−c, and 29a−d (R_1 Monomers). Preparation of Imide 5. Method A. A solution of oxazolidone **12** (7.09 g, 40.0 mmol) in THF (80 mL) was cooled to −78 °C and treated with *n*-butyllithium (26.3 mL, 1.6 M in hexanes). After the solution was stirred at the same temperature for 10 min, an acid chloride (46.0 mmol) was added, and the resulting solution was warmed to 23 °C over 30 min. The reaction mixture was then quenched with saturated aqueous $NaHCO_3$ (80 mL) and concentrated in vacuo to remove the organic solvents. The resulting aqueous residue was extracted with Et_2O (200 mL × 2), and the combined organic layers were washed with water (100 mL × 1) and brine (100 mL × 1), and then dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (10 → 20% EtOAc in hexanes) afforded imide **5** in quantitative yield.

Method B. A mixture of oxazolidone **12** (32.61 g, 184.0 mmol), carboxylic acid (184.0 mmol), and 2-chloro-1-methylpyridinium iodide (61.13 g, 239.2 mmol) in CH_2Cl_2 (350 mL) was cooled to 0 °C and treated with triethylamine (56.4 mL, 404.8 mmol) and 4-(dimethylamino)pyridine (4.50 g, 36.8 mmol). After the reaction mixture was stirred at 23 °C for 1.5 h, hexanes (350 mL) were poured into it. The resulting slurry was passed through a pad of silica gel, eluted with 40% EtOAc in hexanes. The eluted solution was concentrated in vacuo, and the resulting solid was recrystallized from EtOAc−hexanes or the resulting residue was purified by column chromatography (79−94%).

Preparation of Allyl Alcohols. Nonfunctionalized Side Chains. A solution of imide **5** (40.00 mmol) in CH_2Cl_2 (80 mL) was treated with di-*n*-butylboron trifluoromethanesulfonate (42.0 mL, 1.0 M in CH_2Cl_2) at 0 °C. After the solution was stirred at the same temperature for 20 min, diisopropylethylamine (7.66 mL, 44.0 mmol) was added dropwise. After being stirred at 0 °C for 20 min, the resulting mixture was cooled to −78 °C. Acrolein (4.81 mL, 72.0 mmol), which had been passed through a pad of neutral alumina, was added to the solution, and the mixture was stirred at −78 °C for 20 min, followed by 0 °C for 20 min. The mixture was then quenched with phosphate buffer (60 mL, pH 7.0) and MeOH (300 mL), followed by 30% H_2O_2 (40 mL) at 0 °C. After the mixture was stirred at 0 °C for 1 h, the organic solvents were removed in vacuo. The resulting aqueous residue was extracted with EtOAc (200 mL × 2), and the combined organic layers were washed with saturated aqueous NH_4Cl/30% NH_3 (5:1, 200 mL) and brine (100 mL), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (10 → 25% EtOAc in hexanes) afforded allyl alcohols **13** (67−79%).

Functionalized Side Chains. To a solution of imide **5** (169.2 mmol) in CH_2Cl_2 (178 mL) were added diisopropylethylamine (32.4 mL, 177.6 mmol) and then di-*n*-butylboron trifluoromethanesulfonate (177.6 mL, 1.0 M in CH_2Cl_2) at −78 °C. The solution was stirred at 0 °C for 30 min before being cooled to −78 °C. Acrolein (20.3 mL, 304.5 mmol) was then slowly added to the solution at −78 °C, and the resulting solution was stirred at the same temperature for 20 min and at −50 °C for 20 min. The reaction was quenched by the addition of pH 7.0 buffer (254 mL), MeOH (900 mL), and 30% H_2O_2 (169 mL) at −50 °C and stirred at 0 °C for at least 1 h. The organic solvents were removed in vacuo, and the resulting aqueous residue was extracted with EtOAc (750 mL × 2). The same workup as above afforded allyl alcohols **13** (55−76%).

Preparation of Diols 14. A solution of allyl alcohol **13** (20.0 mmol) in Et_2O (70 mL) was treated with lithium borohydride (12.0 mL, 2.0 M in THF) at 0 °C in the air and stirred at the same temperature for 5 min. The reaction mixture was then quenched with saturated aqueous NH_4Cl (30 mL) and 30% NH_3 (10 mL). After the solution was stirred at 23 °C for 1 h, the organic solvents were removed in vacuo, and the aqueous residue was extracted with EtOAc (100 mL × 2). The combined organic layers were then washed with brine (100 mL × 1), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (0.3% MeOH and 30 → 60% EtOAc in hexanes) afforded diol **14** (81−90%) as an oil and imide **12** (90%) as a solid.

Preparation of Silyl Ethers 15. A solution of diol **14** (10.0 mmol) and triethylamine (1.74 mL, 12.5 mmol) in CH_2Cl_2 (20 mL) was treated with *tert*-butyldimethylsilyl chloride (1.73 g, 11.5 mmol) at 0 °C and stirred at 23 °C for 8 h. The reaction mixture was then quenched with phosphate buffer (20 mL, pH 7.0) and concentrated in vacuo to remove the organic solvent. The resulting aqueous residue was extracted with Et_2O (100 mL × 1), and the organic layer was washed with brine (50 mL × 1), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (4 → 8% EtOAc in hexanes) afforded silyl ether **15** (80−89%) as an oil.

Preparation of Trityl Ethers 19 and 25. A solution of diol **14** (25.6 g, 96.9 mmol) and pyridine (10.2 mL, 126 mmol) in CH_2Cl_2 (200 mL) was treated with trityl chloride (30.3 g, 107 mmol) at 0 °C and stirred at the same temperature for 2 h. The reaction mixture was then quenched with EtOH (2 mL) and poured into Et_2O/EtOAc (800 mL/400 mL). The resulting mixture was washed with aqueous $KHSO_4$ (0.3 M, 100

mL × 1), water (70 mL × 1), and brine (70 mL × 1). The organic layer was dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (8 → 20% EtOAc in hexanes) afforded the trityl ether (88−91%) as an oil.

Preparation of Benzoic Acid 22. A solution of ethyl ester **19** (19.5 g, 38.5 mmol) in THF/MeOH (270 mL/180 mL) was treated with aqueous LiOH (77 mL, 2.0 M) and aqueous H_2O_2 (18 mL, 30%) at 0 °C and stirred at 46 °C for 12 h. After the reaction solution was cooled to 0 °C, aqueous sodium sulfite was added to the solution until a KI test was negative. The organic solvents were evaporated in vacuo, and EtOAc (400 mL) and NaH_2PO_4 (250 mL, 1.0 M) were added to the resulting aqueous mixture. After the layers were separated, the aqueous layer was extracted with EtOAc (200 mL × 1). The combined organic layers were washed with water (100 mL × 1) and brine (100 mL × 1), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (70% EtOAc in hexanes) afforded benzoic acid **22** (18.0 g, 98%) as a colorless oil.

Preparation of Benzamides 23b,c. To the mixture of benzoic acid **22** (4.89 g, 10.2 mmol) and 2-chloro-1-methylpyridinium iodide (3.92 g, 15.3 mmol) in CH_2Cl_2 (26 mL) was added diisopropylethylamine (5.34 mL, 30.7 mmol) at 0 °C, and the resulting mixture was stirred at 23 °C for 15 min. The mixture was then cooled to 0 °C and treated with diethyl iminodiacetate (5.00 mL, 27.9 mmol) or methylamine (6.13 mL, 8.0 M in EtOH). The resulting mixture was stirred at 23 °C for 15 min and then poured into EtOAc (150 mL). The organic layer was washed with water (40 mL × 1), aqueous NaH_2PO_4 (40 mL, 1.0 M), and brine (40 mL × 1), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (30 → 40% EtOAc in hexanes for **23c** and 55 → 60% EtOAc in hexanes for **23b**) afforded **23c** (quantitative) as an oil or **23b** (58%) as a white powder.

Preparation of *N*-Alkoxyphthalimides 16, 20a−c, and 26. A solution of the allyl alcohol (1.00 mmol), *N*-hydroxyphthalimide (196 mg, 1.20 mmol), and triphenylphosphine (315 mg, 1.20 mmol) in THF (6.0 mL) was treated with diethyl azodicarboxylate (189 μL, 1.20 mmol) at 0 °C and warmed to 23 °C. After the solution was stirred at the same temperature for 45 min, the solvent was removed in vacuo and the residue passed through a plug of silica gel, eluted with 20% EtOAc in hexanes. The eluant was concentrated and the residue purified by column chromatography (EtOAc in hexanes) to afford the *N*-alkoxyphthalimide (67−97%).

Preparation of Azide 27a. A solution of alkyl bromide **26** (6.38 mmol) in DMF (15 mL) was treated with sodium azide (31.9 mmol) and tetrabutylammonium iodide (3.21 mmol), and the resulting mixture was stirred at 23 °C for 12 h. EtOAc (250 mL) was poured into the solution, and the mixture was washed with water (200 mL). The aqueous layer was extracted with EtOAc (200 mL), and the combined organic layers were washed with brine (250 mL), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (20% EtOAc in hexanes) afforded azide **27a** (3.32 g, 91%) as an oil.

Preparation of Malonate 27b. A solution of sodium hydride (60 wt %, 40.3 mmol) in DMF (10 mL) was treated with diethyl malonate (50.1 mmol) at 0 °C and stirred at 23 °C for 30 min. A solution of alkyl bromide **26** (9.35 mmol) and tetrabutylammonium iodide (4.74 mmol) in DMF (10 mL) was then added at 23 °C, and the mixture was stirred at the same temperature for 12 h. EtOAc (200 mL) was then added, and the mixture was washed with brine (100 mL). The aqueous layer was extracted with EtOAc (200 mL), and the combined organic extracts were washed with aqueous brine (3 × 300 mL), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (20 → 30% EtOAc in hexanes) afforded malonate **27b** (7.65 g, crude) as an oil. A portion of the material was purified by PTLC for further characterization. After deprotection of the phthalimide and reprotection with 2-nitrobenzensulfonyl, the isolated yield was 53% for three steps.

Preparation of Methylenedioxyphenol Ether 27c. After a mixture of 3,4-methylenedioxyphenol (9.83 mmol) and cesium carbonate (7.30 mmol) in DMF (5 mL) was stirred at 23 °C for 1 h, a solution of alkyl bromide **26** (4.78 mmol) in DMF (5 mL) was added to the solution at 23 °C and stirred at the same temperature for 12 h. EtOAc (250 mL) was then added, and the organic layer was washed with brine (250 mL × 2) and aqueous potassium carbonate (0.1 M, 100 mL), dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (1% triethylamine and 10% EtOAc in hexanes) afforded methylenedioxyphenol ether **27c** (1.58 g, 50%) as an oil.

Preparation of *N*-Methylsulfonamide 27d. To *N*-methyl *p*-tolulenesulfonamide (18.97 mmol) was added potassium *tert*-butoxide in THF (1.0 M, 15.2 mL) at 0 °C, and the resulting solution was stirred at 23 °C for 30 min. A solution of alkyl bromide **26** (7.97 mmol) and 4-(dimethylamino)pyridine in DMF (10 mL) and THF (5 mL) was added to the solution at 23 °C, and the mixture was stirred at the same temperature for 16 h. The organic solvent was removed in vacuo, and the resulting solution was extracted with EtOAc (150 mL × 2). The combined organic layers were washed with brine (150 mL), dried over $MgSO_4$, filtered, and concentrated. Purification of the residue by column chromatography (10 → 30% EtOAc in hexanes) afforded *N*-methylsulfonamide **27d** (5.76 g, quantitative) as an oil.

Preparation of *N*-Alkoxysulfonamides 17, 21a−c, and 28a−d. A solution of the *N*-alkoxyphthalimide (1.00 mmol) in CH_2Cl_2 (4.0 mL) was treated with methylamine (0.38 mL, 8.0 M in EtOH) and stirred at 23 °C for 15 min in the air. The solvents were then evaporated in vacuo, and the residue was azeotroped with benzene 3 times to remove methylamine. The residue was dissolved in CH_2Cl_2 (4.0 mL), and 2,4,6-collidine (172 μL, 1.30 mmol) was added to the solution. The resulting mixture was cooled to 0 °C and treated with 2- or 4-nitrobenzenesulfonyl chloride (266 mg, 1.20 mmol). After being stirred at the same temperature for 15 min, the reaction mixture was quenched with diethylamine (10 μL, 0.1 mmol) and then poured into EtOAc (20 mL). The organic layer was washed with aqueous $KHSO_4$ (10 mL, 0.1 M) and then brine, dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (EtOAc in hexanes) afforded the *N*-alkoxysulfonamide (26−93% and 98% (crude)).

Preparation of Alcohols 18. A solution of *N*-alkoxysulfonamide **17** (1.00 mmol) in THF (5.0 mL) in a polypropylene vessel was treated with HF−pyridine (0.6 mL) at 0 °C and stirred at 23 °C for 10 h. The reaction mixture was diluted with Et_2O (5 mL) and quenched with saturated $NaHCO_3$ solution. After removal of the organic solvents in vacuo, the resulting aqueous residue was extracted with EtOAc (50 mL × 2). The combined organic layers were washed with aqueous $KHSO_4$ (50 mL, 0.1 M), water, and brine and then dried over Na_2SO_4, filtered, and concentrated. Purification of the residue by column chromatography (0.2% MeOH and 20 → 30% EtOAc in hexanes) afforded primary alcohol **18** (65−73%).

(2*S*,3*S*)-18c: $R_f = 0.23$ (30% EtOAc in hexanes); $[\alpha]^{24}_D$ −102.5° ($c = 1.0$, benzene); IR (thin film) $\lambda_{max} = 3500$ (br, NH), 3220 (br, OH), 1532, 1349, 1172 cm^{-1}; ^{1}H NMR (500 MHz, 1% MeOD in C_6D_6, 23 °C) $\delta = 7.88$ (ddd, 2H, $J = 9.0, 2.0, 2.0$ Hz, SO_2Ar), 7.73 (ddd, 2H, $J = 9.0, 2.0, 2.0$ Hz, SO_2Ar), 7.18 (dd, 2H, $J = 7.2, 7.0$ Hz, Ph meta), 7.14−7.11 (m, 2H, Ph ortho), 7.04 (t, 1H, $J = 7.2$ Hz, Ph para), 5.74 (ddd, 1H, $J = 17.5, 10.2, 7.7$ Hz, −C*H*═CH_2), 5.26 (dd, 1H, $J = 17.5, 1.5$ Hz, −CH═CH_2 cis), 5.19 (dd, 1H, $J = 10.2, 1.5$ Hz, −CH═CH_2 trans), 4.71 (dd, 1H, $J = 7.7, 7.5$ Hz, −C*H*ONHSO$_2$Ar), 3.66 (dd, 1H, $J = 11.5, 4.0$ Hz, −CH_2OH), 3.47 (dd, 1H, $J = 11.5, 5.5$ Hz, −CH_2OH), 2.74 (dd, 1H, $J = 13.5, 4.5$ Hz, −CH_2Ph), 2.55 (dd, 1H, $J = 13.5, 10.0$ Hz, −CH_2Ph), 2.03 (m, 1H, −C*H*CH$_2$OH); ^{13}C NMR (125 MHz, C_6D_6, 23 °C) $\delta = 150.5, 142.4, 140.1, 134.7, 129.4, 129.3, 128.7, 128.2, 126.5, 124.0, 121.2, 89.2, 59.8, 46.1, 33.2$, one peak was obscured by benzene; ES$^-$ HRMS calcd for $C_{18}H_{19}N_2O_6S$ ($M - H^+$) 391.0964, found 391.0948.

Preparation of Alcohols 24a−c and 29a−c. A solution of the *N*-alkoxysulfonamide (1.00 mmol) in Et_2O (2.5 mL) was treated with formic acid (2.5 mL) and either ethanethiol (1.50 mmol) or mercaptoacetic acid (2.00 mmol) at 23 °C. After the solution was stirred at the same temperature for 2 h, the organic solvents were removed in vacuo. Purification of the residue by column chromatography (0.3% MeOH, 30 → 60% EtOAc in hexanes) afforded the primary alcohols (29−82%).

(2*S*,3*S*)-24a: $R_f = 0.33$ (60% EtOAc in hexanes); $[\alpha]^{24}_D$ +81.3° ($c = 1.0$, benzene); IR (thin film) $\lambda_{max} = 3550$ (br, NH), 3270 (br, OH), 1713 (C═O), 1542, 1366, 1280, 1179 cm^{-1}; ^{1}H NMR (500 MHz, 1% MeOD in C_6D_6, 23 °C) $\delta = 8.09$ (d, 2H, $J = 8.1$ Hz, Ar ortho to ester), 7.97 (d, 1H, $J = 8.0$ Hz, −SO_2Ar), 7.06 (d, 2H, $J = 8.1$ Hz, Ar meta to ester), 6.88 (d, 1H, $J = 8.0$ Hz, −SO_2Ar), 6.72 (dd, 1H, $J =$

8.0, 8.0 Hz, $-SO_2Ar$), 6.54 (dd, 1H, J = 8.0, 8.0 Hz, $-SO_2Ar$), 5.55 (ddd, 1H, J = 17.5, 10.0, 8.0 Hz, $-CH{=}CH_2$), 5.17 (d, 1H, J = 17.5 Hz, $-CH{=}CH_2$ cis), 5.12 (d, 1H, J = 10.0 Hz, $-CH{=}CH_2$ trans), 4.63 (dd, 1H, J = 8.0, 8.0 Hz, $-CHONHSO_2Ar$), 4.12 (q, 2H, J = 7.5 Hz, $-OCH_2CH_3$), 3.57 (dd, 1H, J = 11.5, 3.5 Hz, $-CH_2OH$), 3.32 (dd, 1H, J = 11.5, 4.5 Hz, $-CH_2OH$), 2.54 (dd, 1H, J = 13.7, 4.5 Hz, $-CH_2Ar$), 2.48 (dd, 1H, J = 13.7, 10.0 Hz, $-CH_2Ar$), 1.85 (m, 1H, $-CHCH_2OH$), 1.02 (t, 3H, J = 7.5 Hz, $-OCH_2CH_3$); ^{13}C NMR (125 MHz, C_6D_6, 23 °C) δ = 166.2, 148.4, 145.7, 144.5, 134.4, 134.3, 132.7, 132.2, 130.5, 130.0, 129.8, 129.5, 127.0, 125.3, 121.6, 89.1, 60.7, 59.6, 45.8, 33.2, 14.2; ES$^+$ HRMS calcd for $C_{21}H_{24}N_2O_8SNa$ (M + Na$^+$) 487.1151, found 487.1159.

(2*S*,3*S*)-29a: R_f = 0.30 (50% EtOAc in hexanes); $[\alpha]^{24}_D$ +94.4° (*c* = 1.5, benzene); IR (thin film) λ_{max} = 3550 (br, NH), 3260 (br, OH), 2099 (N_3), 1722, 1542, 1392, 1360, 1178 cm^{-1}; ^{1}H NMR (500 MHz, 1% MeOD in C_6D_6, 23 °C) δ = 8.01 (dd, 1H, J = 7.7, 1.0 Hz, $-SO_2Ar$), 6.84 (dd, 1H, J = 7.7, 1.0 Hz, $-SO_2Ar$), 6.77 (ddd, 1H, J = 7.7, 7.7, 1.0 Hz, $-SO_2Ar$), 6.52 (ddd, 1H, J = 7.5, 7.7, 1.0 Hz, $-SO_2Ar$), 5.45 (ddd, 1H, J = 17.2, 10.0, 8.2 Hz, $-CH{=}CH_2$), 5.13 (dd, 1H, J = 17.2, 1.0 Hz, $-CH{=}CH_2$ cis), 5.08 (dd, 1H, J = 10.0, 1.0 Hz, $-CH{=}CH_2$ trans), 4.50 (dd, 1H, J = 8.2 Hz, $-CHONHSO_2Ar$), 3.59 (dd, 1H, J = 11.5, 4.5 Hz, $-CH_2OH$), 3.40 (dd, 1H, J = 11.5, 5.0 Hz, $-CH_2OH$), 2.66−2.62 (m, 2H, $-CH_2N_3$), 1.43 (br m, 1H, $-CHCH_2OH$), 1.32−1.23 and 1.21−1.12 (m, 4H, $-CH_2CH_2CH_2N_3$); ^{13}C NMR (125 MHz, C_6D_6, 23 °C) δ = 134.5, 134.3, 132.8, 132.2, 128.5, 125.1, 120.8, 89.1, 61.0, 51.3, 43.4, 41.4, 26.5, 24.1; ES$^+$ HRMS calcd for $C_{14}H_{19}N_5O_6SNa$ (M + Na$^+$) 408.0945, found 408.0962.

(B) Preparation of Homoallyl Alcohols 3 (R_2 Monomers). To a solution of (+)-*B*-methoxydiisopinocamphenylborane (21.17 g, 66.9 mmol) in Et_2O (45 mL) was added a solution of allylmagnesium bromide (60.8 mL, 1 M in Et_2O) dropwise at 0 °C, and the flask was then allowed to warm to 23 °C over 1 h. The mixture was cooled to −78 °C and treated with methyl 4-formylbenzoate (9.99 g, 60.8 mmol). The reaction mixture was allowed to warm to 23 °C over 3 h, at which time the solvents were removed in vacuo. A 1:1 mixture of aqueous NaOH (3.0 M) and MeOH (150 mL) was added to the flask at 0 °C, 30% H_2O_2 (20 mL) was then added dropwise, and the mixture was stirred at 23 °C for 72 h. The MeOH was then removed in vacuo, and additional H_2O (100 mL) and aqueous NaOH (50 mL, 3.0 M) were added to the flask. The mixture was washed with EtOAc (50 mL × 2), and the aqueous extracts were acidified to pH 2 with concentrated HCl and extracted with EtOAc (100 mL × 2). The combined organic layers were then washed with brine (70 mL × 2) and concentrated to give the homoallyl alcohol (58−73%).

The subsequent ester hydrolysis and amide formation for **3f** was accomplished by the same procedure as for ethyl ester **19**.

(*S*)-3f: R_f = 0.53 (100% Et_2O); $[\alpha]^{25}_D$ −12.9° (*c* = 1.0, benzene); IR (thin film) λ_{max} = 3500 (br, OH), 1744 (C=O), 1642, 1209, 1191 cm^{-1}; ^{1}H NMR (500 MHz, 1% MeOD in C_6D_6, 23 °C) δ = 7.48 (d, 1H, J = 7.7 Hz, Ar ortho to amide), 7.05 (d, 1H, J = 7.7 Hz, Ar meta to amide), 5.61 (ddd, 1H, J = 18.0, 7.5, 7.5 Hz, $-CH{=}CH_2$), 4.92−4.88 (m, 2H, $-CH{=}CH_2$ cis and trans), 4.31−4.28 (m, 3H, $-CHOH$ and $-NCH_2CO_2Et$), 3.98 (s, 2H, $-NCH_2CO_2Et$), 3.89−3.76 (m, 4H, $-OCH_2CH_3$), 2.25−2.15 (m, 2H, $-CH_2CH{=}CH_2$), 0.88−0.77 (m, 6H, $-OCH_2CH_3$); ^{13}C NMR (125 MHz, C_6D_6, 23 °C) δ = 172.1, 171.4, 169.2, 169.0, 147.2, 134.8, 134.1, 127.3, 126.1, 117.6, 72.8, 61.1, 61.0, 60.3, 51.8, 50.0, 47.9, 44.0, 13.9; ES$^+$ HRMS calcd for $C_{19}H_{25}NO_6Na$ (M + Na$^+$) 386.1580, found 386.1565.

Preparation of Cinnamate Esters. To a solution of **3i** (7.36 g, 32.4 mmol), palladium(II) acetate (0.18 g, 0.82 mmol), and triorthotolyl phosphine (0.74 g, 2.42 mmol) in degassed NMP (50 mL) were added degassed methyl acrylate (29.2 mL, 323.9 mmol) and triethylamine (6.70 mL, 48.1 mmol). After the mixture was heated to 90 °C for 1.5 h, and then to 110 °C for 2 h, palladium(II) acetate (0.084 g, 0.37 mmol) and triorthotolyl phosphine (0.55 g, 1.82 mmol) were added, and the suspension was stirred at 110 °C for 1 h. Et_2O (400 mL) and water (250 mL) were poured into the reaction mixture, and the layers were separated. The organic layer was washed with brine (250 mL), dried with $MgSO_4$, and concentrated. Purification of the residue by column chromatography (15 → 20% EtOAc in hexanes) afforded cinnamate **3g** (3.25 g, 43%) as an oil.

(*S*)-3g: R_f = 0.15 (20% EtOAc in hexanes); $[\alpha]^{25}_D$ −11.3° (*c* = 1.0, benzene); IR (thin film) λ_{max} = 3500 (br, OH), 1704 (C=O), 1638, 1322, 1195, 1175 cm^{-1}; ^{1}H NMR (400 MHz, 1% MeOD in C_6D_6, 23 °C) δ = 7.75 (d, 1H, J = 16.0 Hz, $-CH{=}CHCO_2CH_3$), 7.37 (s, 1H, Ar ortho $-CH{=}CHCO_2CH_3$ and $-CHOH$), 7.21 (d, 1H, J = 7.2 Hz, Ar para $-CHOH$), 7.06−6.99 (m, 2H, J = 7.2 Hz, Ar meta and para $-CH{=}CHCO_2CH_3$), 6.43 (d, 1H, J = 16.0 Hz, $-CH{=}CHCO_2CH_3$), 5.75 (m, 1H, $-CH{=}CH_2$), 5.01−4.95 (m, 2H, $-CH{=}CH_2$), 4.54 (dd, 1H, J = 5.6, 1.6 Hz, C*H*OH), 3.86 (s, 3H, $-CH{=}CHCO_2CH_3$), 2.47−2.31 (m, 2H, $-CH_2CH{=}CH_2$); ^{13}C NMR (100 MHz, C_6D_6, 23 °C) δ = 166.9, 145.5, 144.9, 134.7, 134.7, 128.9, 127.1, 126.0, 118.4, 118.0, 73.1, 51.4, 44.3, one peak was obscured by benzene; CI$^+$ HRMS calcd for $C_{14}H_{20}NO_3$ (M + NH_4^+) 250.1443, found 250.1431.

(C) Solid-Phase Library Synthesis. Preparation of Trityl Ethers 4. To the polystyrene trityl chloride resin (4.00 g, 8.10 mmol) in CH_2Cl_2 (8.0 mL) in a polypropylene tube was added a solution of the primary alcohol (1.00 mmol) and pyridine (1.97 mL, 24.3 mmol) in CH_2Cl_2 (14.0 mL) at 0 °C. After the reaction vessel was shaken at 23 °C for 24 h, the resin was washed with DMF (20 mL × 2) and CH_2Cl_2 (20 mL × 3) before it was dried in vacuo. The completion of the reaction was confirmed by monitoring the solution by thin-layer chromatography and noting the absence of the starting alcohol. The resin was then treated with a solution of MeOH (1.4 mL) or EtOH (1.4 mL) and pyridine (1.97 mL, 24.3 mmol) in CH_2Cl_2 (14.0 mL) at 23 °C for 24 h. The resin was washed with DMF (20 mL × 2) and CH_2Cl_2 (20 mL × 3) and dried in vacuo. The resulting loading level was estimated to be 0.20 mmol/g (144 mg of the resin was treated with the cleaving solution and 10.3 mg of (2S,3R)-**24b** was isolated, which indicates that the actual loading level for this compound was approximately 0.16 mmol/g).

Preparation of the Acetamide. To resin-bound azide **29a** (508 mg, 0.13 mmol) was added thiolacetic acid (4.0 mL). After the reaction vessel was shaken at 23 °C for 1 h, the resin was washed with DMF (5 mL × 3) and CH_2Cl_2 (5 mL × 3) before it was dried in vacuo. LC−MS analysis confirmed the presence of the desired product in high purity (expected mass 401.1, experimental $(m/z)^+$ = 402.1 for M + H$^+$).

Splitting the Resin. A slurry of the resin was prepared in 40% CH_2Cl_2 in NMP, and a portion of the mixture was distributed into each reaction vessel. As a result, each contained approximately 30 mg (6.0 μmol) of the resin.

Preparation of Dienes 2. (i) For Homoallyl Alcohols R_2 = 3a−c. To the solution of di-*tert*-butyl azodicarboxylate (737 mg, 3.20 mmol) and Ph_3P (800 mg, 3.05 mmol) in THF (2.90 mL) at −10 °C was added an aliphatic alcohol (2.31 mmol) in THF (2.90 mL). After being stirred at the same temperature for 1 min, 240 μL of the solution was added to each starting material (20 reactions for each R_2 monomer). The resulting mixture was shaken at 23 °C for 30 min, and the resin was washed with DMF (2 mL × 2), CH_2Cl_2 (2 mL × 2), and THF (2 mL × 1) and dried in vacuo.

(ii) For Benzylic Homoallyl Alcohols R_2 = 3d−h. To the solution of a benzylic alcohol (1.94 mmol) and Ph_3P (560 mg, 2.13 mmol) in THF (6.93 mL) at −50 °C was added diisopropyl azodicarboxylate (482 μL, 2.33 mmol). After the solution was stirred at the same temperature for 1 min, a 300-μL aliquot was added to each starting material (20 reactions for each R_2 monomer). The resulting mixture was shaken at 23 °C for 20 min, and the resin was washed with DMF (2 mL × 2), CH_2Cl_2 (2 mL × 2), and THF (2 mL × 1) and dried in vacuo.

Preparation of Tetrahydrooxazepines 31. (i) For R_1 = 18a−c, R_2 = 3a−d. A solution of bis(tricyclohexylphosphine)benzylidene ruthenium(IV) dichloride (182 mg, 222 μmol) in degassed CH_2Cl_2 (26.4 mL) was prepared, and 0.5 mL of the solution was added to each reaction vessel (48 reactions) at 23 °C. The sealed vessels were shaken at 23 °C for 1 h before the resin was washed with DMF (2 mL × 2), CH_2Cl_2 (2 mL × 2), and THF (2 mL × 1) and dried in vacuo.

(ii) For R_1 = 18a−c, R_2 = 3e−h and R_1 = 24a−c, R_2 = 3a−h and R_1 = 29a−d, R_2 = 3a−h. A solution of bis(tricyclohexylphosphine)benzylidene ruthenium(IV) dichloride (4.29 g, 5.21 mmol) in degassed CH_2Cl_2 (145 mL) was prepared, and 0.5 mL of the solution was added to each reaction vessel (272 reactions) at 23 °C. The sealed

vessels were shaken at 23 °C for 1 h, and the resin was washed with DMF (2 mL × 2), CH_2Cl_2 (2 mL × 2), and THF (2 mL × 1) before it was dried in vacuo.

After this step, 5 mg (ca. 1.0 μmol) of the resin from each well was transferred to another 96-well plate, and the compound was released from solid support by 1% TFA/5% triisopropylsilane in CH_2Cl_2 at 23 °C for 1 min.

Preparation of Alkoxyamines 32. For the remaining steps, each well contained approximately 5 mg of the starting material resin.

(i) For R_1 = 18a−c, R_2 = 3a−f (72 Compounds). A solution of ethanedithiol (0.50 mL, 6.0 mmol) and DBU (0.45 mL, 3.0 mmol) in CH_2Cl_2 (12.0 mL) was prepared at −78 °C, 130 μL of the solution was added to each vessel, and the resulting mixture was shaken at 23 °C for 10 min before the resin was washed with DMF (2 mL × 2) and CH_2Cl_2 (2 mL × 2). The same procedure was repeated three times, and then the resin was dried in vacuo.

(ii) For R_1 = 18a−c, R_2 = 3g,h (24 Compounds). A solution of β-mercaptoethanol (210 μL, 3.0 mmol) and DBU (225 μL, 1.5 mmol) in CH_2Cl_2 (6.0 mL) was prepared at −78 °C, 195 μL of the solution was added to each vessel, and the resulting mixture was shaken at 23 °C for 10 min before the resin was washed with DMF (2 mL × 2) and CH_2Cl_2 (2 mL × 2). The same procedure was repeated three times. A solution of DBU in NMP (0.50 mL, 0.25 M) was then added to the resin, and the resulting mixture was shaken for 20 min before the resin was washed with NMP (0.5 mL × 1). The same procedure was repeated until Michael adducts were not detected by HPLC analysis (three times). The resin was then washed with DMF (2 mL × 2) and CH_2Cl_2 (2 mL × 2) and dried in vacuo.

(iii) For R_1 = 24a−c and 29a−d, R_2 = 3a−h (224 Compounds). A solution of thiophenol (2.78 mL, 27.0 mmol) and DBU (2.03 mL, 13.5 mmol) in NMP (54 mL) was prepared at 0 °C, 195 μL of the solution was added to each vessel, and the mixture was shaken at 23 °C for 30 min. The resin was then washed with NMP (0.5 mL × 1), and the same procedure was repeated twice. The resin was then washed with DMF (0.2 mL × 2) and CH_2Cl_2 (0.2 mL × 2) and dried in vacuo.

Preparation of Amides 33. (i) For R_1 = 18a−c, 24a−c, and 29a−d, R_2 = 3a−c (120 Compounds). Fmoc-6-aminohexanoic acid (915 mg, 2.59 mmol) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (1.08 g, 2.85 mmol) were dissolved in NMP (21.6 mL), and diisopropylethylamine (0.99 mL, 5.70 mmol) was added to the solution at 0 °C. A 70-μL aliquot of the resulting solution was added to each well, and the mixture was shaken at 23 °C for 1 h. The resin was then washed with DMF (0.2 mL × 2), CH_2Cl_2 (0.2 mL × 2), and THF (0.2 mL × 1) and dried in vacuo.

(ii) For R_1 = 18a−c, 24a−c, and 29a−d, R_2 = 3d−h (200 Compounds). Fmoc-6-aminohexanoic acid (2.33 g, 6.60 mmol) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (2.64 g, 6.93 mmol) were dissolved in NMP (9.2 mL) and CH_2Cl_2 (10.0 mL), and diisopropylethylamine (2.3 mL, 13.2 mmol) was added to the solution at 0 °C. A 91-μL aliquot of the resulting solution was added to each well, and the mixture was shaken at 23 °C for 18 h. The resin was then washed with DMF (0.2 mL × 2), CH_2Cl_2 (0.2 mL × 2), and THF (0.2 mL × 1) and dried in vacuo.

Preparation of Primary Amines 34. A 150-μL aliquot of piperidine in DMF (150 μL, 20% v/v) was added to each well at 23 °C and drained slowly at the same temperature over 5 min. The same procedure was repeated three times, and the resin was then washed with DMF (0.2 mL × 3), CH_2Cl_2 (0.2 mL × 3), and toluene (0.2 mL × 1) and dried in vacuo.

Synthesis of Amides 35. To a mixture of AP1867 (749 mg, 1.08 mmol) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (493 mg, 1.30 mmol) in CH_2Cl_2 (10.1 mL) and NMP (15.4 mL) was added diisopropylethylamine (0.45 mL, 2.59 mmol) at 0 °C. After the solution was stirred at 23 °C for 5 min, a 70-μL aliquot was added to each well. The reaction vessels were shaken at 23 °C for 3 h, and the resin was washed with CH_2Cl_2 (0.2 mL × 3), DMF (0.2 mL × 3), CH_2Cl_2 (0.2 mL × 3), and THF (0.2 mL × 1) and dried *in vacuo*.

Synthesis of Primary Alcohols 36. A solution of 1% TFA and 5% triisopropylsilane in CH_2Cl_2 (0.18 mL) was passed through the resin three times at 23 °C for 1 min each. The eluted solution was collected in 96-well plates, and the organic solvents were evaporated under the stream of nitrogen gas. The residue was then dried in vacuo.

(D) Determination of the Permeability of the Heterodimeric Compounds to Mammalian Cells. Cell Lines and Tissue Culture. The human HT1080 fibrosarcoma cell line 41-5 was obtained from ARIAD Gene Therapeutics, Inc. The 41-5 cell line contains a retrovirally integrated Z12-IL2-SEAP reporter and is stably transfected with the CMV-driven transcription factor expression construct pCEN-Fv3p65/Z1Fv3/Neo.[20] The cells were propagated in minimal essential medium (GIBCO) containing 10% (vol/vol) fetal calf serum, non-essential amino acids, penicillin/streptomycin, and Geneticin.

Assay. HT1080 cells plated in a 96-well dish (1×10^4 cells/well) in 200 μL of medium were treated with both AP1889 (final concentration, 10 nM) and a library compound (final concentration, 100 nM). After incubation for 21 h, the cells were viable, and 100 μL of the medium from each well was transferred to another 96-well dish. The transferred medium was then incubated at 67 °C for 1.5 h and cooled to 23 °C. A 100-μL aliquot of 4-methylumbelliferyl phosphate solution (1.2 mM, pH 10.0) was added to each well, and the resulting mixture was incubated at 37 °C for 8 h. The fluorescence intensity was recorded using a Titertek Fluoroskan II (ICN) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm.[11]

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Supporting Information Available: Spectral characterization data for all intermediates produced by solution-phase synthesis and LC−MS traces of 40 of the final heterodimeric compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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