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Structural Determination of NSC 670224, Synthesis of Analogues and Biological Evaluation

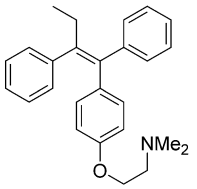
Nathaniel B. Zuckerman,[a] Andrew S. Myers,[a] Tiffani K. Quan,[b, c] Walter M. Bray,[a] R. Scott Lokey,[a] Grant A. Hartzog,[b] and Joseph P. Konopelski*[a]

In the search for new lead compounds for drug discovery, small-molecule and natural product libraries of known biologically active compounds are being investigated by high-throughput (HT) screening methods for potential new uses. One such screening protocol employs the budding yeast *Saccharomyces cerevisiae* as a platform that is both convenient and broadly applicable. Yeast and mammalian cells have many conserved cellular processes, yeast deletion mutants are widely available, and profiling samples across the genome-wide yeast deletion libraries can facilitate target identification.[1–3]

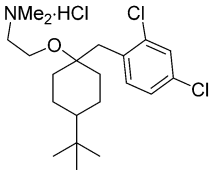
Previously published work identified a group of 46 known compounds as toxic towards *S. cerevisiae* through the use of a HT yeast halo assay[4] on the US National Cancer Institute (NCI) Diversity Set I Mechanistic Set and Natural Products Set, comprising a total of 3104 synthetic and natural compounds.[5] Genome-wide chemical sensitivity profiling[6] of these hits against the library of yeast deletion mutants highlighted two compounds that showed very similar profiles: NSC-180973 (tamoxifen) and NSC 670224. In human cells, a good correlation between the two compounds in the NCI 60 cell line screen (correlation coefficient = 0.55) was found using the COMPARE algorithm[7] (see Figure S1 in the Supporting Information). These data suggest that the compounds could share a target in human cells that, based on the yeast profiling data, is not necessarily related to the estrogen receptor (ER).

Tamoxifen has been a highly effective treatment for ER-positive breast cancer for over 30 years, and its mechanism of action in these cases is relatively well understood. However, in some cases, tamoxifen has been shown to be effective against ER-negative breast cancer, which seems to indicate an alternative mechanism of action or target. Furthermore, the genome of *S. cerevisiae* does not encode any protein with significant homology to the human ER. Although there are reports connecting the bioactivity of tamoxifen in yeast to calcium signaling,[8,9] our analysis pointed toward an alternative pathway that accounts for the activity of both tamoxifen and NSC 670224. To our knowledge, there are no published reports on the bioactivity of NSC 670224.

Analysis of the library sample of NSC 670224 indicated a pure single isomer that was not stereochemically defined in the structure deposited on the NCI database. Our interest in probing the possible mechanistic link between tamoxifen and NSC 670224 led us to undertake the synthesis of both diastereomers of the nominal structure (a 2,4-dichloro arene) for stereochemical determination and biological assay. Herein, we describe the results that have led to: 1) the identification of the correct structure of the compound found in the NCI library; 2) the production of a targeted library of NSC 670224 derivatives for biological evaluation; 3) yeast lethality data for compounds prepared in this study; 4) the preparation of a compound related to NSC 670224 that can be derivatized for protein target identification.



NSC 180973 (tamoxifen)



NSC 670224

Initially, we explored the reaction of Grignard reagents with ketal **1** as a direct route to the desired structure class, as had been reported in the patent literature.[10] Commercially available benzylmagnesium chloride was reacted with **1** and provided the expected diastereomeric mixture of primary alcohols *cis*- and *trans*-**2**, which were separated by chromatography (Scheme 1). Single-crystal X-ray analysis of the primary alcohol eluted first confirmed the structure to be *cis*-**2**. Mesylation, displacement with aqueous dimethylamine,[11] and formation of the hydrochloride salt were performed for each diastereomer of **2** to give *cis*- and *trans*-**3**. However, this synthetic route, which delivered the first compounds for our structure–activity relationship (SAR) study, could not be employed for the synthesis of NSC 670224 as the requisite dichlorobenzyl halide undergoes extensive homocoupling during the formation of the corresponding Grignard reagent.

A longer, but ultimately more fruitful, procedure was developed from the reaction between 2,4-dichlorobenzyl chloride and magnesium in the presence of lithium chloride and zinc

[a] *N. B. Zuckerman, A. S. Myers, W. M. Bray, Prof. R. S. Lokey, Prof. J. P. Konopelski*
Department of Chemistry and Biochemistry
University of California, Santa Cruz (UCSC)
PSB 230, 1156 High Street, Santa Cruz, CA 95064 (USA)
E-mail: joek@ucsc.edu

[b] *Dr. T. K. Quan, Prof. G. A. Hartzog*
Department of Molecular, Cell and Developmental Biology
University of California, Santa Cruz (UCSC)
Sinsheimer Labs, 1156 High Street, Santa Cruz, CA 95064 (USA)

[c] *Dr. T. K. Quan*
Department of Biochemistry and Biophysics
University of California, San Francisco (UCSF)
Genentech Hall, MC 2140600
600 16th Street, Suite S222, San Francisco, CA 94158 (USA)

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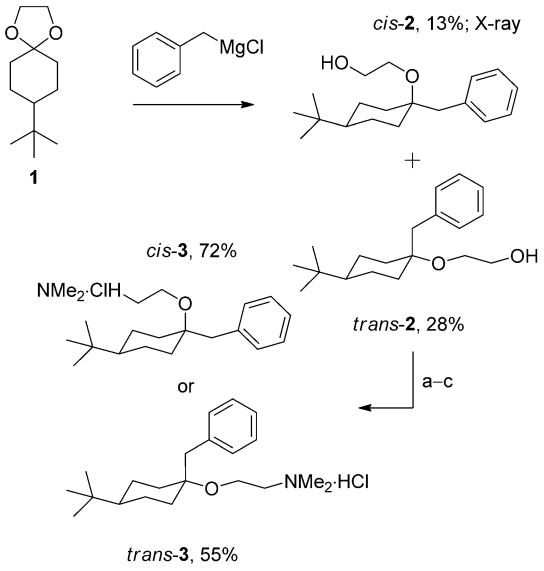
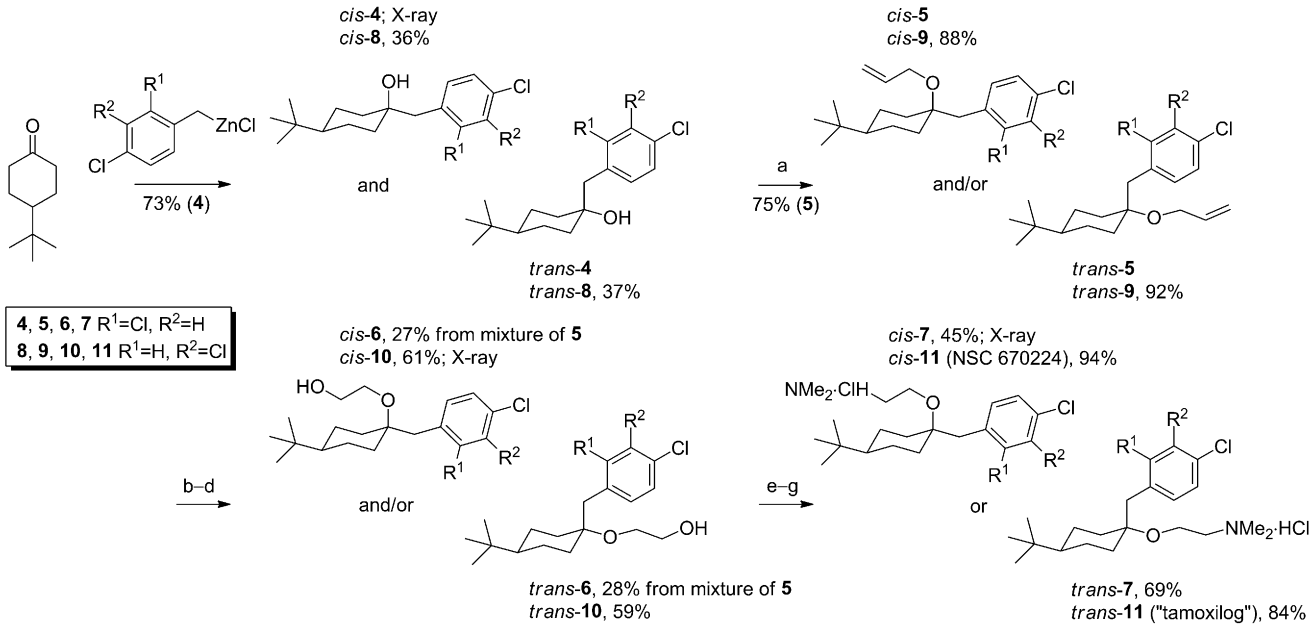


Scheme 1. Grignard addition to ketal. *Reagents and conditions*: a) MsCl, Et_3N, CH_2Cl_2, RT, 2 h; b) aq $HNMe_2$, refluxing THF, 16 h; c) HCl gas, Et_2O, RT, 2 min, 55% (three steps).

chloride, as defined by Knochel and co-workers (Scheme 2).[12] The resulting organozinc reagent reacted cleanly with 4-*tert*-butylcyclohexanone to afford tertiary alcohols *cis*- and *trans*-**4** is approximately a 1:1 ratio. Although these isomers were separable by column chromatography, they were taken through to the next steps as a mixture. As with compound **2**, X-ray analysis confirmed that *cis*-**4** was the first isomer to elute. Allylation of the tertiary alcohols with potassium hydride, sodium iodide and allyl bromide[13] gave compounds **5** in 75% yield. The mixture of *cis*- and *trans*-**5** was subjected to catalytic osmylation using osmium tetroxide/*N*-methylmorpholine-*N*-oxide (NMO), followed by oxidative cleavage of the crude diols with sodium periodate/silicon dioxide,[14] and reduction of the crude aldehyde with sodium borohydride to give primary alcohols *cis*- and *trans*-**6**, which proved easily separable by column chromatography. Mesylation, displacement, and formation of the hydrochloride salt were conducted on each primary alcohol as described for the benzyl compounds **2** to provide *cis*- and *trans*-**7**.

Unexpectedly, the analytical data of NSC 670224 matched neither synthetic diastereomer of **7** (Figure S2 in the Supporting Information), although mass spectrometry indicated that compounds **7** were isomers of the NCI library sample. Particularly relevant were the differences observed in the aromatic region of the ^1H NMR spectra, which led to the consideration of the 3,4-dichloro regioisomer as the true structure of NSC 670224. The structure of *cis*-7 was confirmed by single-crystal X-ray analysis, and this diastereomer was again the first to elute during chromatography.

Fortunately, the route devised for the synthesis of **7** could be used without difficulty for the synthesis of the 3,4-dichloro isomers as depicted in Scheme 2. Thus, *cis*- and *trans*-**8** were formed in 73% yield as a separable 1:1 mixture of isomers, of which *trans*-**8** was highly crystalline. Each individual isomer of **8** was subjected to the sequence of reaction conditions that led to the preparation of **7**, including formation of allyl ethers **9** and primary alcohols **10** prior to installation of the terminal dimethylamine functionality that defines the final targets, *cis*- and *trans*-**11**. Single-crystal X-ray analysis of *cis*-**10** established the identity of both isomers. Analytical data support the true structure of NSC 670224 to be *cis*-**11**.



Scheme 2. Synthesis of *cis*/*trans*-**7** and **11**. *Reagents and conditions*: a) KH, NaI, 1,2-dimethoxyethane, 0 °C, 5 min; then allyl bromide, 0 °C→RT, overnight; b) For **5**: cat. OsO_4, NMO (1.5 equiv), dioxane, RT in dark, overnight; For **9**: AD-mix-β, *t*-BuOH/H_2O (1:1), RT in dark, overnight; c) $NaIO_4$, SiO_2, CH_2Cl_2, RT, 3 h; d) $NaBH_4$, MeOH, 0 °C, 30 min; e) MsCl, Et_3N, CH_2Cl_2, RT, 2 h; f) aq $HNMe_2$, refluxing THF, 16 h; g) HCl gas, Et_2O, RT, 2 min.

Table 1. Evaluation of tamoxifen and synthetic derivatives in a lethality assay on wild-type *S. cerevisiae*.

Compd	LC_{50}[a] [μM]
cis-**3**	26
trans-**3**	29
cis-**7**	6.7
trans-**7**	2.5
NSC 670224 (*cis*-**11**)	3.2
trans-**11** ("tamoxilog")	2.3
NSC 180973 (tamoxifen)	4.1

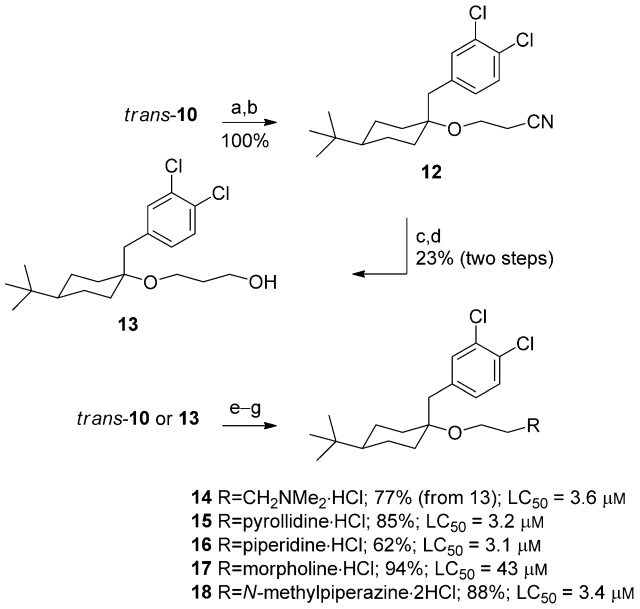
[a] Lethal concentration (LD): the test compound concentration required to kill 50% of yeast.

These synthetic efforts afforded a collection of compounds that varied in stereochemistry and identity of the benzyl substituent. Yeast biological activity studies (Table 1 and Figure S3 in the Supporting Information) verified the more potent activity of the original library structure *cis*-**11** as compared with both isomers of **3** and *cis*-**7**. The lack of chlorine substituents on the benzyl group is shown to significantly lower the activity, as seen for compounds **3**. Within the most active compounds, the *trans* isomer is consistently more active than the *cis*. Hence, *trans*-**11** was found to be more potent than both NSC 670224 and tamoxifen in yeast, and therefore was given the name "tamoxilog" to identify both its activity profile and its structure as the origin of our further SAR studies.

To that end, the decision was made to probe the importance of the choline chain, since this functionality is common to both NSC 670224 and tamoxifen. The synthesis of homologue **14** began by mesylation of *trans*-**10** and displacement with potassium cyanide in dimethyl sulfoxide (DMSO) as depicted in Scheme 3.[15] Nitrile **12** was partially reduced to the aldehyde with diisobutylaluminum hydride (DIBAL), and the crude aldehyde was further reduced to primary alcohol **13** in poor yield over two steps.[16] Compound **13** was aminated under conditions similarly to those used for **6** and **10** to give homologue **14**. The synthesis of derivatives **15**–**18** utilized the respective secondary amines in displacement of the mesylate formed from *trans*-**10**, followed by formation of their hydrochloride salts.

Compounds **14**–**18** were evaluated in a yeast lethality assay (Scheme 3 and Figure S3 in the Supporting Information). None of the derivatives matched the activity of *trans*-**11**, although both homologue **14** and pyrollidine analogue **15** are quite active. This activity profile is supported by growth inhibition studies, which measure yeast growth at a set concentration of agent (5 μM) over a particular time course (Figure 1). Morpholino derivative **17** is nearly devoid of activity, showing yeast growth similar to that observed for the DMSO standard. *trans*-**11** ("tamoxilog"), homologue **14** and pyrollidine derivative **15** all present highly active profiles.

The activity data clearly indicate that important contributions are made by both the choline chain and the chlorinated benzyl substituent. Consequently, it seemed reasonable to suggest that modification of the *tert*-butyl group would not substantially affect the biological activity. This was important since



Scheme 3. Synthesis and activity against *S. cerevisiae* of analogues **14**–**18**. *Reagents and conditions*: a) MsCl, Et_3N, CH_2Cl_2, RT, 2 h; b) KCN, DMSO, 65 °C, 2 h; c) DIBAL, hexane, −78 °C, 30 min; then RT 3 h; d) $NaBH_4$, MeOH, 0 °C, 30 min; e) MsCl, Et_3N, CH_2Cl_2, RT, 2 h; f) aq $HNMe_2$, refluxing THF, 16 h; g) HCl gas, Et_2O, RT, 2 min.

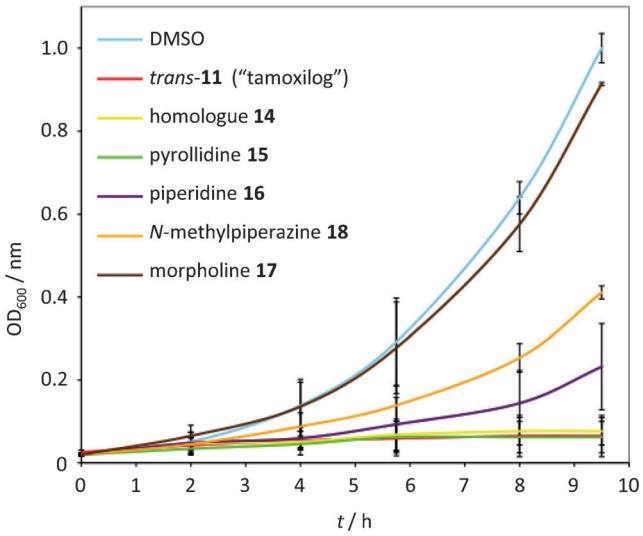
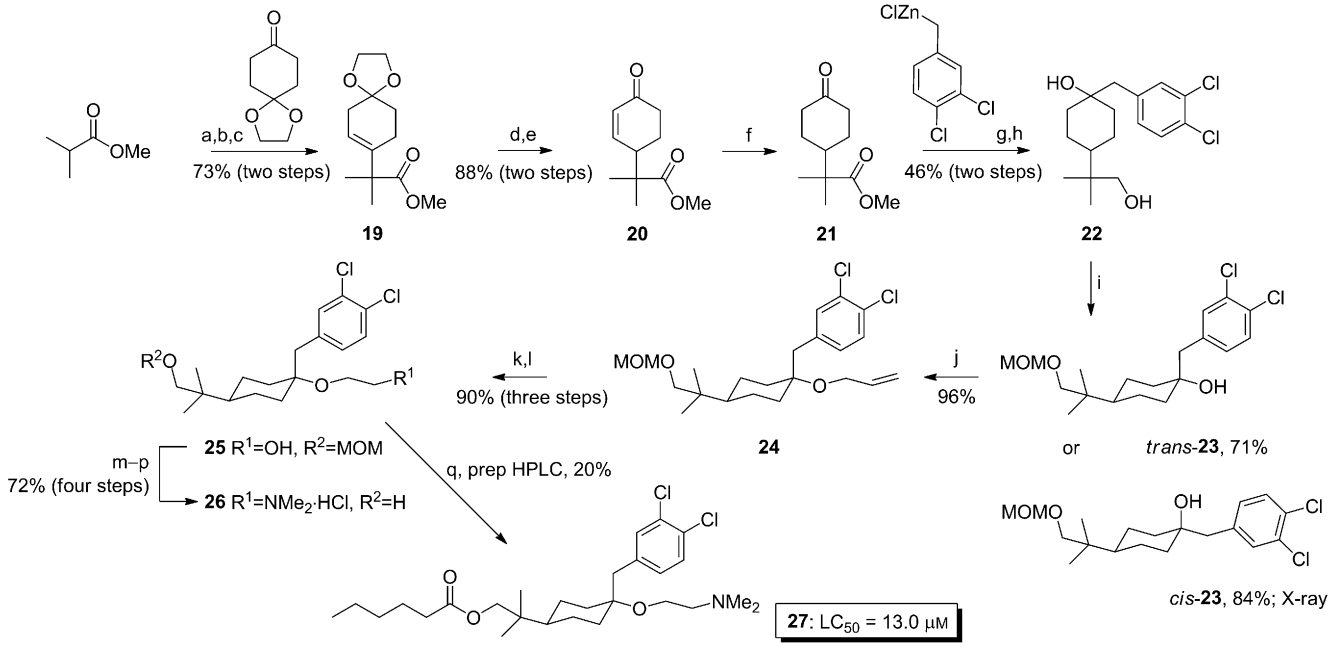


Figure 1. Average growth of wild-type yeast cells over 9.5 h at a test compound concentration of 5 μM, as measured by optical density (OD) of 600 nm.

there is great interest in determining the cellular target of *trans*-**11**, which requires a biologically active derivative for the synthesis of a molecular probe. An alcohol functionality was chosen as the handle on *trans*-**11** from which probe molecules could be prepared through an ester linkage that, if necessary, can be cleaved under mildly basic conditions.[17] To test this hypothesis, a simple hexanoate ester derivative of *trans*-**11** was prepared and subjected to biological analysis. Synthesis of the potential probe molecule is outlined in Scheme 4.



Scheme 4. Synthesis of modified *trans*-**11** ("tamoxilog"). *Reagents and conditions*: a) LDA, −78 °C, 20 min; b) ketone, −78 °C, 2 h; c) $SOCl_2$, pyridine, 0 °C→RT, overnight; d) PPTS, acetone/water (9:1) reflux, 2 d; e) DBU, CH_2Cl_2, reflux, 2 h; f) Pd/C, H_2, 1 atm; g) 3,4-dichlorobenzyl zincate, THF, RT, overnight; h) $LiAlH_4$, 0 °C→RT, 3 h; i) MOMCl, Hunig's base, RT, overnight; j) KH, NaI, 1,2-dimethoxyethane, 0 °C, 5 min; then allyl bromide, 0 °C→RT, overnight; k) OsO_4 (2 mol %), NMO (1.5 equiv) RT, overnight; then $NaIO_4$, RT, 4 h; l) $NaBH_4$, MeOH, 0 °C, 30 min; m) MsCl, Et_3N, CH_2Cl_2, RT, 2 h; n) aq $HNMe_2$, refluxing THF, 16 h; o) HCl, MeOH, H_2O, RT, 24 h; p) HCl gas, Et_2O, RT, 2 min; q) EDCI, 4-DMAP; $C_5H_{12}CO_2H$, 0 °C→RT, 16 h.

Using the method of Engel and Schexnayder,[18] deprotonation of methyl isobutyrate with lithium diisopropylamide (LDA) and reaction with monoethylene glycol-protected 1,4-diketocyclohexane gave the corresponding alcohol. After work-up, the crude alcohol was dehydrated with thionyl chloride in pyridine to give alkene **19** in 73% yield over two steps. Attempts to reduce the tri-substituted double bond of compound **19** via catalytic hydrogenation were unsuccessful, even at elevated pressures. However, deprotection of the ketal with catalytic *p*-toluenesulfonic acid (PTSA) gave a mixture of the deprotected tri-substituted alkene together with the corresponding α,β-unsaturated isomer, which could be reduced at atmospheric pressure with hydrogen and palladium on carbon (Pd/C). Due to the appearance of a significant amount of an unknown side product under catalytic PTSA conditions, compound **19** was deprotected with pyridinium *p*-toluenesulfonate (PPTS) in refluxing acetone/water.[19] The crude alkene was isomerized in refluxing dichloromethane with 2,3,4,6,7,8,9,10-octahydropyrimidol[1,2-*a*]azepine (DBU)[20] to give α,β-unsaturated ketone **20** in 88% yield over two steps. Finally, reduction of **20** with Pd/C and hydrogen (1 atm) gave keto-methyl ester **21** in 96% yield.

It was originally envisioned that the methyl ester would mask the alcohol through the remaining steps. However, introduction of the aryl substituent using conditions described by Knochel et al.[12] provided the desired tertiary alcohols as an inseparable mixture. Therefore, the crude mixture was reduced with lithium aluminum hydride to give a mixture of diols **22**, which could be separated efficiently by column chromatography. The *cis* and *trans* diastereomers of **22** were reacted with methyl chloromethyl ether to give mono-methoxymethyl ether (MOM)-protected *cis*-**23** and *trans*-**23** in 84% and 71% yield, respectively. Although it was envisioned that only the *trans* diastereomer would be taken through to the desired material, *cis*-**23** was found to give X-ray-quality crystals, thus allowing the stereochemical determination of both species. Allylation of *trans*-**23** with potassium hydride, allyl bromide and catalytic sodium iodide gave allyl ether **24** in 96% yield. Conversion of the allyl ether to primary alcohol **25** was conducted over three steps in 90% yield in accordance with a procedure published by Cossy et al.[21] Mesylation of **25** and displacement of the product with dimethylamine gave crude MOM-protected amine, which was deprotected in a mixture of hydrochloric acid/methanol/water. The oil that remained after deprotection was passed through a deactivated silica gel column (1% triethylamine in the mobile phase) to give the free amine. The amine was converted to the hydrochloride salt with hydrogen chloride gas in diethyl ether, and after recrystallization, desired alcohol **26** was obtained in 72% over four steps. Alcohol **26** could be coupled with commercially available hexanoic acid in *N*,*N*-dimethylformamide with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and catalytic 4-dimethylaminopyridine (DMAP). Purification with preparative HPLC gave desired hexanoate ester **27** in 20% yield. Biological evaluation of compound **27** showed the compound to be active, with an LC_{50} value of 13.0 μM (Figure S4 in the Supporting Information).

The synthetic work described in this paper has clarified the erroneous structural annotation of NSC 670224 in the NCI library. In addition, the preparation of a focused library of derivatives and a molecule (**26**) capable of use for the preparation

of an affinity probe has set the stage for biological evaluation and target identification of *trans*-**11** ("tamoxilog"), the *trans* isomer of NSC 670224. Probe synthesis and full biological studies are ongoing and will be disclosed in due course.

Experimental Section

Full details of synthetic protocols are provided in the Supporting Information. CCDC 862851, 862852, 862853, 862854, 862855 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Yeast growth assays: Wild-type cells (strain BY4741)[22] were grown in yeast extract peptone dextrose (YPD) media at 30 °C. Cells were counted by using a hemocytometer, and cultures were diluted to 8×10^5 cells mL^{-1}. The desired concentration of tamoxifen, *trans*-**11** ("tamoxilog") and related compounds (5 μM) or DMSO was added, and cultures were incubated at 30 °C with rotation. Optical density at 600 nm (OD_{600}) was recorded every 1.5–2 h, starting at the time of dilution.

LC_{50} values: Strain BY4741 (195 μL) was plated into a 96-well plate at an OD_{600} value of 0.1. Test compound (5 μL) was added to each well and two-fold serial dilutions of each compound were tested. OD_{600} readings were taken every 30 min with an EnVision plate (PerkinElmer). After 16 h of incubation, the OD_{600} was plotted against concentration in Prism (GraphPad) to generate a dose–response curve and calculate the LC_{50} value.

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Keywords: affinity probes · drug discovery · high-throughput screening · medicinal chemistry · tamoxifen

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