Peptidomimetics Hot Paper

International Edition: DOI: 10.1002/anie.201510557
German Edition: DOI: 10.1002/ange.201510557

# Stapled Peptides with γ-Methylated Hydrocarbon Chains for the Estrogen Receptor/Coactivator Interaction

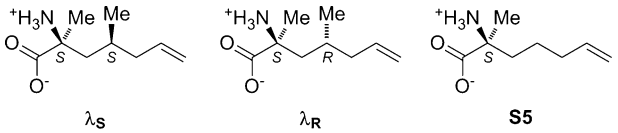
*Thomas E. Speltz, Sean W. Fanning[+], Christopher G. Mayne[+], Colin Fowler, Emad Tajkhorshid, Geoffrey L. Greene, and Terry W. Moore**

***Abstract:*** *"Stapled" peptides are typically designed to replace two non-interacting residues with a constraining, olefinic staple. To mimic interacting leucine and isoleucine residues, we have created new amino acids that incorporate a methyl group in the γ-position of the stapling amino acid S5. We have incorporated them into a sequence derived from steroid receptor coactivator 2, which interacts with estrogen receptor α. The best peptide ($IC_{50}$ = 89 nM) replaces isoleucine 689 with an S-γ-methyl stapled amino acid, and has significantly higher affinity than unsubstituted peptides (390 and 760 nM). Through X-ray crystallography and molecular dynamics studies, we show that the conformation taken up by the S-γ-methyl peptide minimizes the syn-pentane interactions between the α- and γ-methyl groups.*

Several strategies exist for stabilizing or mimicking[1] biologically active peptide sequences and secondary structures with small molecules or constrained peptides.[2] Among the most widely used mimics of α-helices are "stapled" peptides.[3] They feature a side chain to side chain olefin crosslink that may imbue peptides with enhanced conformational stability,[4] metabolic stability, and, somewhat controversially, cell permeability.[5] To synthesize stapled peptides, two or more strategically chosen residues of a native peptide sequence are replaced with non-natural α-methyl-α-alkenyl amino acids. Ring-closing metathesis forms a macrocycle between the $i$ and $i+3$, $i+4$, or $i+7$ positions.[6] As the constraint may interfere with the ability of the peptide to bind to its receptor, stapled peptides are typically designed so that the constraint is placed on a non-interacting face of an α-helix.[3a] Recently, others have reported successfully replacing interacting helical residues with a staple.[7] Although it lacked the branching functionality of valine, leucine, and isoleucine, the staple had the ability to fill protein surface grooves. As we show in this work, incorporating hydrophobic functionality at the constraint may more accurately mimic native sequences to increase affinity, and, importantly, it may also further stabilize bioactive conformations.

Phillips et al. reported an early example of replacing interacting residues with a hydrocarbon staple.[7a] The crystal structure of stapled peptide PFE-SP2 bound to estrogen receptor α (ERα) showed that an $i$, $i+4$ hydrocarbon staple can replace isoleucine and leucine residues on the binding face of a steroid receptor coactivator 2 (SRC2) peptide. This replacement yields an increase in α-helicity and affinity. SRC2 interacts with the surface of ERα over two turns of an α-helix using an ILXXLL motif (X is any amino acid).[8] Although recalcitrant, this protein–protein interaction has been well-investigated[9] to treat endocrine therapy-resistant breast cancers. Recently identified ERα mutants that are constitutively active and implicated in metastases have brought renewed focus to this therapeutically important interaction.[10]

We designed stapled peptides that incorporate branched stapling residues as functionalized constraints. Specifically, we designed amino acids based on stapling amino acid S5 that incorporate a methyl group in the γ-position to mimic the branched hydrophobic amino acids Ile689 and Leu693 of the $I_{689}LXXLL_{694}$ motif of SRC2 (Scheme 1). As S5 contains an α-methyl group for helical stability, incorporation of a γ-methyl group establishes 1,5-interactions, which, when appropriately positioned, could bolster the helical conformations imposed by the constraint. We synthesized the requisite amino acids $\lambda_R$ and $\lambda_S$ by joining one of Schöllkopf's bis(lactim) ethers with enantioenriched branched alkenyl side chains, which were synthesized using Evans' *N*-acyloxazolidinone chemistry (see the Supporting Information).[11] These amino acids, in combination with S5, were incorporated into residues 687–697 of SRC2. Solid-phase peptide synthesis and ring-closing metathesis were carried out as previously reported, and four stapled peptides containing $\lambda_R$/$\lambda_S$ and/or S5 were successfully




***Scheme 1.*** Branched stapling amino acids.

[*] T. E. Speltz, Prof. T. W. Moore
Department of Medicinal Chemistry and Pharmacognosy and UI Cancer Center
University of Illinois at Chicago
833 S. Wood St., Chicago, IL 60612 (USA)
E-mail: twmoore@uic.edu
Dr. S. W. Fanning,[+] C. Fowler, Prof. G. L. Greene
The Ben May Department for Cancer Research
The University of Chicago
Chicago, IL 60637 (USA)
Dr. C. G. Mayne,[+] Prof. E. Tajkhorshid
Beckman Institute for Advanced Science and Technology
The University of Illinois at Urbana-Champaign
Urbana, IL 61801 (USA)
[+] These authors contributed equally to this work.
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201510557.

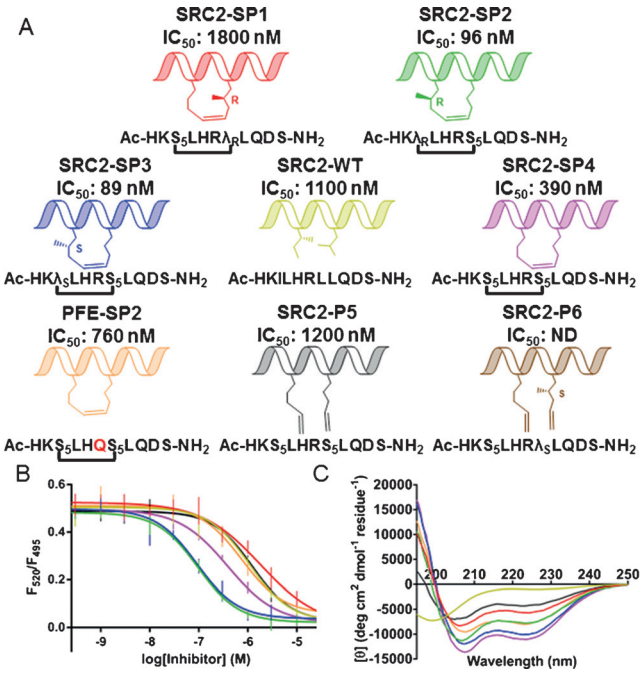



***Figure 1.*** A) Peptides used in this study. B) TR-FRET dose-response curves for the inhibition of ERα/SRC3. C) CD measurements were taken in 45 mM phosphate buffer at pH 7.4 with 10% MeOH. SRC2-WT yellow, SRC2-SP1 red, SRC2-SP2 green, SRC2-SP3 blue, SRC2-SP4 magenta, PFE-SP2 orange, SRC2-P5 gray, SRC2-P6 brown.

synthesized (see Figure 1A). The *Z* alkene configuration was consistent with the $^1$H NMR H–C=C–H coupling constants of 10–11 Hz (see the Supporting Information). SRC2-P6 failed to undergo ring-closing metathesis, even under forcing conditions, suggesting that substituting the $i+4$ stapling residue with *R*-configured γ-substituted moieties results in conformations that are non-productive for ring closure.

A time-resolved fluorescence resonance energy transfer (TR-FRET) assay (Figure 1B) was used to measure the interaction of a steroid receptor coactivator 3 (SRC3) fragment with the ERα ligand-binding domain.[12] In this assay, the wild-type peptide has an $IC_{50}$ of 1100 nM. The unfunctionalized stapled peptide SRC2-SP4 has an $IC_{50}$ of 390 nM. This peptide is analogous to PFE-SP2, which was described by Phillips et al., but has a wild-type Q→R substitution, which increases the affinity by a factor of two. Epimers SRC2-SP2 and SRC2-SP3 were the most active, showing a twelvefold increase in potency compared to the wild-type peptide. SRC2-SP1, designed to incorporate a branched stapling residue to replace conserved Leu693, displayed minimal activity. Aside from the TR-FRET assay, surface plasmon resonance (see the Supporting Information) was used to obtain dissociation constants for SRC2-SP1 (530 nM), SRC2-SP2 (42 nM), and SRC2-SP3 (39 nM).

Circular dichroism (CD) analysis of the peptides (Figure 1C) indicates that the wild-type sequence is disordered and that the stapled peptide SRC2-SP4 adopts an α-helical conformation in solution. The CD spectrum for SRC2-SP1 shows that $\lambda_R$ substitution at Leu693 negatively impacts α-helicity; however, $\lambda_S$ substitution at Ile689 (SRC2-SP3) maintains helicity as does $\lambda_R$ substitution at Ile689 (SRC2-SP2), albeit to a lesser extent. The observation that the addition of methyl groups may positively impact affinity while having a slightly negative effect on helicity may imply that constructive interactions with the surface of the receptor are more important for affinity than locking in a helical conformation.

We obtained co-crystal structures of SRC2-SP1, -SP2, -SP3, -SP4, and -P5 bound to the ligand-binding domain of a constitutively active Y537S ERα mutant. In all cases, the peptides bind in a similar α-helical conformation,[13] occupy the hydrophobic groove, and make contacts with the so-called "charge clamp"—flanking Lys and Glu residues that align complementarily with the inherent dipole of the coactivator helix. The most notable difference is that the stapled peptides display a 1.2 Å shift towards the Glu end of the charge clamp, as compared to the wild-type protein (Figure 2A–C). The γ-$CH_3$ group of SRC2-SP1 occupies the same region as Leu693 (Figure 2A), and it occupies a pseudo-equatorial conformation to alleviate unfavorable *syn*-pentane strain between the α- and γ-methyl groups (Figure 2D). The resulting orientation of the γ-methyl group increases contact with Ile358 of ERα, which may disrupt its interaction with the Lys end of the charge clamp. Minimizing *syn*-pentane interactions at this position also substantially alters the $\chi_1$ torsion angle (−44°) at Leu693 relative to the more helical stapled peptides (i.e., $\chi_1 = +61°$ for SRC2-SP3; see Figure 2A,C). Analogous to SRC2-SP1, minimization of *syn*-pentane interactions is seen with the γ-methyl group of SRC2-SP3, but instead of opposing the predominant conformation, the *S*-γ-methyl group reinforces a high-affinity conformation (Figure 2D). Furthermore, the γ-methyl group occupies the same region as Ile689 in the wild-type sequence (Figure 2C). The γ-methyl group of SRC2-SP2 also occupies this same space (Figure 2B,D), even though the methyl groups have the opposite configuration. The change in the $\chi_2$ torsion angles between these two peptides is approximately 120°, which can explain how this observation is possible (Figure 2D).

We carried out molecular dynamics (MD) studies on SRC2-SP1, -SP2, -SP3, and -SP4 bound to ERα using the NAMD2[14] simulation package with trajectory analysis performed in VMD.[15] The structural ensembles confirmed the strong influence of *syn*-pentane interactions on the conformation taken up by the staple. In particular, the dihedral angles between position 693 in SRC2-SP1 and SRC2-SP2, -SP3, and -SP4 are opposite in sign, with substantially more fluctuation at this position in SRC2-SP1 (see Figure 3A). In agreement with the X-ray structure, the simulations suggest that SRC2-SP2 adopts a pseudo-eclipsed conformation of −90° at position 689. ERα accommodates the branching methyl group of the $\lambda_S$ residue at position 689, but the $\lambda_R$ residue at position 693 introduces a steric clash with Ile358 of the protein and induces a substantial shift in peptide positioning (Figure 3C). We also carried out MD studies on SRC2-SP1, -SP2, -SP3, and -SP4 in solution in the absence of ERα. These data confirm that the observed $\chi_1$ torsion angles in solution correlate well with the observed angles in the crystal structure, with the caveat that SRC2-SP4 shows stable

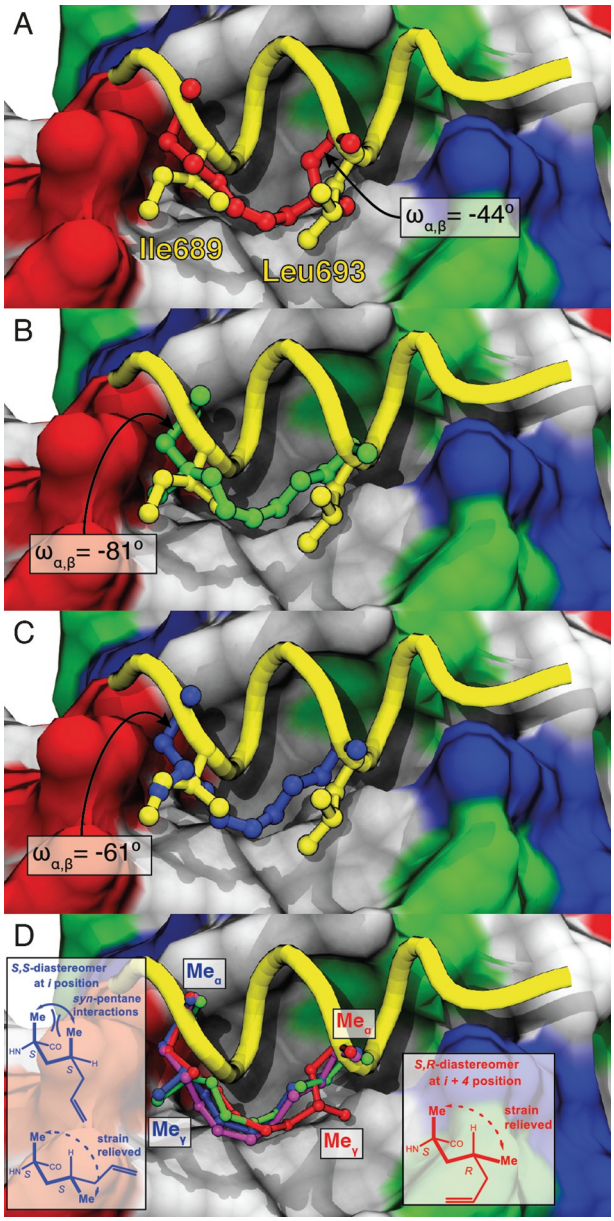



***Figure 2.*** X-ray co-crystal structures of peptides bound to ERα (surface: red acidic, blue basic, white nonpolar, green polar). A) SRC2-SP1 (red, PDB 5DXB), B) SRC2-SP2 (green, PDB 5HYR), and C) SRC2-SP3 (blue, PDB 5DX3) superimposed onto SRC2-WT (yellow, PDB 3ERD). Torsion angles ($\omega_{\alpha,\beta}$) about the Cα–Cβ bond at position Leu693 are given. D) Hydrocarbon staples of SRC2-SP1 (red), SRC2-SP3 (blue), and SRC2-SP4 (magenta, PDB 5DXE) superimposed onto the backbone of SRC2-WT (shown as a yellow tube). SRC2-SP1, SRC2-SP2, and SRC2-SP3 adopt conformations to alleviate *syn*-pentane interactions between the α- and γ-methyl groups. The side chains of non-cyclic SRC2-P5 also bind along the hydrophobic shelf (see the Supporting Information).

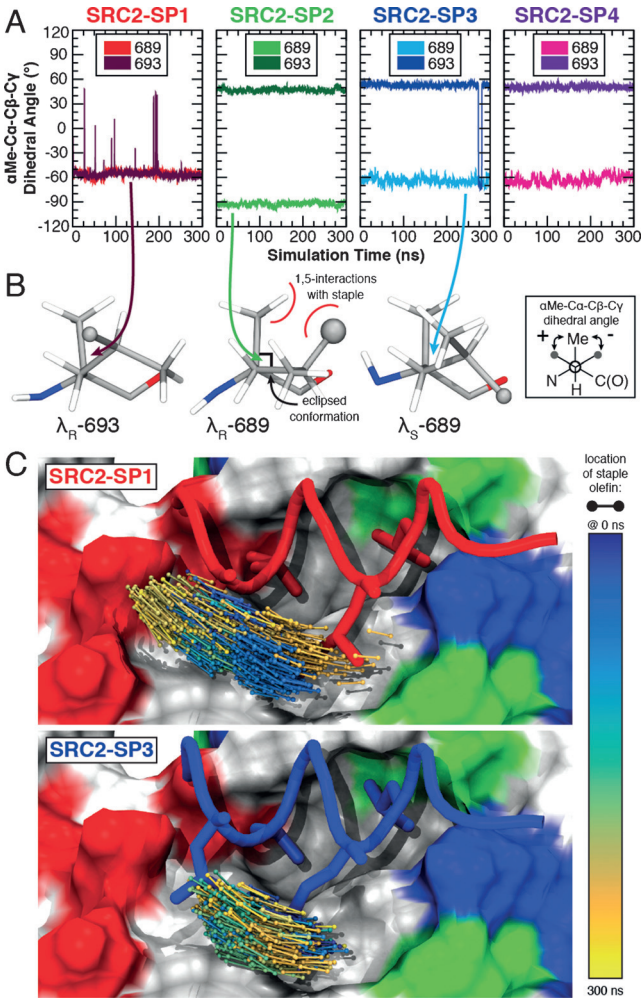



***Figure 3.*** MD simulations of peptides bound to ERα. A) The $\chi_1$ dihedral angle reveals different conformations of staple residues 689 and 693 for peptides SRC2-SP1, SRC2-SP2, and SRC2-SP3. B) The structural conformations of the γ-methyl-substituted residue are shown for the last frame of the simulation. C) The position of the staple shifts substantially during the course of the simulation for SRC2-SP1 (top) and is relatively stable for SRC2-SP3 (bottom).

conformations at both −60° and −90° in solution (see the Supporting Information).

In conclusion, we have created a stapling amino acid, $\lambda_S$, that both mimics native branched side chains and stabilizes a helical conformation. In this study, we have used this amino acid and its epimer, $\lambda_R$, to prepare highly potent inhibitors of the ERα/SRC interaction. We have shown that incorporation of a γ-methyl group in the *R* or *S* configuration at the *i* position of an *i*, *i*+4 stapled peptide is a tolerated modification that allows the hydrocarbon staple to effectively mimic branched hydrophobic residues, although the *S*-methyl group results in a conformation with higher helical content than the *R*-methyl group. The *S*-methyl moiety reinforces an α-helical conformation through minimization of *syn*-pentane interactions. Incorporation of a γ-methyl group at the *i*+4 position in either configuration appears to have a destabilizing effect on α-helicity. Although the design here is for interacting residues, the incorporation of γ-methyl groups may be applicable to non-interacting stapled residues as well. In this regard, the simulated and observed staple geometries achieved with methyl substitution have provided a blueprint for installing γ-methyl groups and other substituents in stapling amino acids for related protein–protein interactions.

## Acknowledgements

This work was funded by the American Association of Colleges of Pharmacy (to T.W.M.), the University of Illinois Cancer Center (to T.W.M.), the Searle Funds at The Chicago Community Trust (to T.W.M. and G.L.G.), and the NIH (P41-GM104601 to E.T.). T.E.S. is funded by training grant T32 AT007533, sponsored by the Office of the NIH Director and the National Center for Complementary and Integrative Health. Supercomputing resources were provided by the NSF (XSEDE allocation MCA06N060 to E.T.). We thank Kathryn Carlson and John Katzenellenbogen (University of Illinois at Urbana-Champaign) for providing reagents for TR-FRET and Hyun Lee (Research Resources Center, UIC), Gerd Prehna, and Ben Ramirez (Center for Structural Biology, UIC) for help with SPR, CD, and NMR studies.

**Keywords:** amino acids · conformational analysis · peptides · peptidomimetics · receptors

**How to cite:** *Angew. Chem. Int. Ed.* **2016**, *55*, 4252 – 4255
*Angew. Chem.* **2016**, *128*, 4324 – 4327

[1] M. Pelay-Gimeno, A. Glas, O. Koch, T. N. Grossmann, *Angew. Chem. Int. Ed.* **2015**, *54*, 8896 – 8927; *Angew. Chem.* **2015**, *127*, 9022 – 9054.

[2] L. Nevola, E. Giralt, *Chem. Commun.* **2015**, *51*, 3302 – 3315.

[3] a) G. L. Verdine, G. J. Hilinski, *Methods Enzymol.* **2012**, *503*, 3 – 33; b) P. M. Cromm, J. Spiegel, T. N. Grossmann, *ACS Chem. Biol.* **2015**, *10*, 1362 – 1375.

[4] C. E. Schafmeister, J. Po, G. L. Verdine, *J. Am. Chem. Soc.* **2000**, *122*, 5891 – 5892.

[5] Q. Chu, R. E. Moellering, G. J. Hilinski, Y.-W. Kim, T. N. Grossmann, J. T. H. Yeh, G. L. Verdine, *MedChemComm* **2015**, *6*, 111 – 119.

[6] Y. W. Kim, T. N. Grossmann, G. L. Verdine, *Nat. Protoc.* **2011**, *6*, 761 – 771.

[7] a) C. Phillips, L. R. Roberts, M. Schade, R. Bazin, A. Bent, N. L. Davies, R. Moore, A. D. Pannifer, A. R. Pickford, S. H. Prior, C. M. Read, A. Scott, D. G. Brown, B. Xu, S. L. Irving, *J. Am. Chem. Soc.* **2011**, *133*, 9696 – 9699; b) C. H. Douse, S. J. Maas, J. C. Thomas, J. A. Garnett, Y. Sun, E. Cota, E. W. Tate, *ACS Chem. Biol.* **2014**, *9*, 2204 – 2209; c) Y. S. Chang, B. Graves, V. Guerlavais, C. Tovar, K. Packman, K.-H. To, K. A. Olson, K. Kesavan, P. Gangurde, A. Mukherjee, T. Baker, K. Darlak, C. Elkin, Z. Filipovic, F. Z. Qureshi, H. Cai, P. Berry, E. Feyfant, X. E. Shi, J. Horstick, D. A. Annis, A. M. Manning, N. Fotouhi, H. Nash, L. T. Vassilev, T. K. Sawyer, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, E3445 – E3454; d) A. Glas, D. Bier, G. Hahne, C. Rademacher, C. Ottmann, T. N. Grossmann, *Angew. Chem. Int. Ed.* **2014**, *53*, 2489 – 2493; *Angew. Chem.* **2014**, *126*, 2522 – 2526; e) S. Baek, P. S. Kutchukian, G. L. Verdine, R. Huber, T. A. Holak, K. W. Lee, G. M. Popowicz, *J. Am. Chem. Soc.* **2012**, *134*, 103 – 106; f) M. L. Stewart, E. Fire, A. E. Keating, L. D. Walensky, *Nat. Chem. Biol.* **2010**, *6*, 595 – 601.

[8] D. M. Heery, E. Kalkhoven, S. Hoare, M. G. Parker, *Nature* **1997**, *387*, 733 – 736.

[9] a) C.-Y. Chang, J. D. Norris, H. Grøn, L. A. Paige, P. T. Hamilton, D. J. Kenan, D. Fowlkes, D. P. McDonnell, *Mol. Cell. Biol.* **1999**, *19*, 8226 – 8239; b) S. Fuchs, H. D. Nguyen, T. T. Phan, M. F. Burton, L. Nieto, I. J. de Vries-van Leeuwen, A. Schmidt, M. Goodarzifard, S. M. Agten, R. Rose, C. Ottmann, L. G. Milroy, L. Brunsveld, *J. Am. Chem. Soc.* **2013**, *135*, 4364 – 4371; c) L. Caboni, D. G. Lloyd, *Med. Res. Rev.* **2013**, *33*, 1081 – 1118; d) N. Gallastegui, E. Estébanez-Perpiñá in *Nuclear Receptors: From Structure to the Clinic* (Eds.: I. J. McEwan, R. Kumar), Springer International Publishing, **2015**, pp. 179 – 203; e) T. W. Moore, C. G. Mayne, J. A. Katzenellenbogen, *Mol. Endocrinol.* **2010**, *24*, 683 – 695.

[10] a) S. Li, D. Shen, J. Shao, R. Crowder, W. Liu, A. Prat, X. He, S. Liu, J. Hoog, C. Lu, L. Ding, O. L. Griffith, C. Miller, D. Larson, R. S. Fulton, M. Harrison, T. Mooney, J. F. McMichael, J. Luo, Y. Tao, R. Goncalves, C. Schlosberg, J. F. Hiken, L. Saied, C. Sanchez, T. Giuntoli, C. Bumb, C. Cooper, R. T. Kitchens, A. Lin, C. Phommaly, S. R. Davies, J. Zhang, M. S. Kavuri, D. McEachern, Y. Y. Dong, C. Ma, T. Pluard, M. Naughton, R. Bose, R. Suresh, R. McDowell, L. Michel, R. Aft, W. Gillanders, K. DeSchryver, R. K. Wilson, S. Wang, G. B. Mills, A. Gonzalez-Angulo, J. R. Edwards, C. Maher, C. M. Perou, E. R. Mardis, M. J. Ellis, *Cell Rep.* **2013**, *4*, 1116 – 1130; b) K. Merenbakh-Lamin, N. Ben-Baruch, A. Yeheskel, A. Dvir, L. Soussan-Gutman, R. Jeselsohn, R. Yelensky, M. Brown, V. A. Miller, D. Sarid, S. Rizel, B. Klein, T. Rubinek, I. Wolf, *Cancer Res.* **2013**, *73*, 6856 – 6864; c) D. R. Robinson, Y. M. Wu, P. Vats, F. Su, R. J. Lonigro, X. Cao, S. Kalyana-Sundaram, R. Wang, Y. Ning, L. Hodges, A. Gursky, J. Siddiqui, S. A. Tomlins, S. Roychowdhury, K. J. Pienta, S. Y. Kim, J. S. Roberts, J. M. Rae, C. H. Van Poznak, D. F. Hayes, R. Chugh, L. P. Kunju, M. Talpaz, A. F. Schott, A. M. Chinnaiyan, *Nat. Genet.* **2013**, *45*, 1446 – 1451; d) W. Toy, Y. Shen, H. Won, B. Green, R. A. Sakr, M. Will, Z. Li, K. Gala, S. Fanning, T. A. King, C. Hudis, D. Chen, T. Taran, G. Hortobagyi, G. Greene, M. Berger, J. Baselga, S. Chandarlapaty, *Nat. Genet.* **2013**, *45*, 1439 – 1445.

[11] a) U. Schöllkopf, *Tetrahedron* **1983**, *39*, 2085 – 2091; b) D. A. Evans, M. D. Ennis, D. J. Mathre, *J. Am. Chem. Soc.* **1982**, *104*, 1737 – 1739.

[12] J. R. Gunther, Y. Du, E. Rhoden, I. Lewis, B. Revennaugh, T. W. Moore, S. H. Kim, R. Dingledine, H. Fu, J. A. Katzenellenbogen, *J. Biomol. Screening* **2009**, *14*, 181 – 193.

[13] K. W. Nettles, J. B. Bruning, G. Gil, J. Nowak, S. K. Sharma, J. B. Hahm, K. Kulp, R. B. Hochberg, H. Zhou, J. A. Katzenellenbogen, B. S. Katzenellenbogen, Y. Kim, A. Joachmiak, G. L. Greene, *Nat. Chem. Biol.* **2008**, *4*, 241 – 247.

[14] J. C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kalé, K. Schulten, *J. Comput. Chem.* **2005**, *26*, 1781 – 1802.

[15] W. Humphrey, A. Dalke, K. Schulten, *J. Mol. Graph.* **1996**, *14*, 33 – 38.

Received: November 13, 2015
Published online: March 1, 2016