



An enthalpic basis of additivity in biphenyl hydroxamic acid ligands for stromelysin-1

Erin M. Wilfong, Yu Du, Eric J. Toone*

Department of Chemistry, Duke University, LSRC B120, Box 90317, Durham, NC 27708, USA

ARTICLE INFO

Article history:

Received 29 October 2011

Revised 29 April 2012

Accepted 8 May 2012

Available online 30 May 2012

Keywords:

Additivity

Fragment based drug design

Stromelysin-1

Matrix metalloproteinase-3

ABSTRACT

Fragment based drug discovery remains a successful tool for pharmaceutical lead discovery. Although based upon the principle of thermodynamic additivity, the underlying thermodynamic basis is poorly understood. A thermodynamic additivity analysis was performed using stromelysin-1 and a series of biphenyl hydroxamate ligands identified through fragment additivity. Our studies suggest that, in this instance, additivity arises from enthalpic effects, while interaction entropies are unfavorable; this thermodynamic behavior is masked by proton transfer. Evaluation of the changes in constant pressure heat capacities during binding suggest that solvent exclusion from the binding site does not account for the dramatic affinity enhancements observed.

© 2012 Elsevier Ltd. All rights reserved.

In 1997, Fesik and co-workers first reported the use of fragment based drug design (FBDD) to generate high affinity inhibitors for stromelysin-1 (matrix metalloproteinase 3).¹ Since then, FBDD has taken its place among most successful approaches to lead compound discovery. FBDD is based on the principle that proteins binding sites can be parsed into multiple low affinity sub-sites. The binding of small libraries (<200 compounds) of small ligands (<150 Da) are evaluated using techniques that identify the binding locus within the larger binding pocket; subsequent chemical linkage of two or more fragments with affinities for adjacent pockets yields high affinity ligands. Over a decade of use, FBDD had yielded high affinity leads for over fifty proteins, at least some of which have progressed to clinical trials.²

Despite its widespread utility, the thermodynamic principles that underlie the success of FBDD are poorly understood. The notion of additivity in ligand binding was first considered by Jencks.³ Although thermodynamic parameters are state functions and are additive in and of themselves, there is no fundamental basis for any simple relationship between the thermodynamic parameters characterizing the interaction of an intact ligand and those of its constituent fragments. Consider the binding of a ligand AB and of its constituent fragments A and B. Any thermodynamic parameter, J , describing the binding of AB is related to the corresponding parameters for the binding of the constitutive fragments through the relationship:

$$\Delta J_{AB} = \Delta J_A + \Delta J_B + \Delta J_i$$

In this construct, ΔJ_i is an interaction energy describing the energetic consequences of physical linkage. Interaction energies are most frequently considered in entropic terms, a trade-off between a 'saving' in losses in translational and rotational entropy that arises through linkage, and an increase in losses in conformational degrees of freedom, as a flexible tether is constrained during binding.

In contrast to this intellectually straightforward construct, the thermodynamic origin of additivity has been investigated experimentally in several systems, including multivalent carbohydrates and metal chelates and found to be an *enthalpic* phenomena,^{4,5} that is in the construct of Jencks both ΔH_i and $T\Delta S_i$ are negative. On the other hand, Borsi et al. recently conducted an additivity analysis using matrix metalloproteinase-12 (MMP12) and determined that additivity in that case was entropically driven and enthalpically opposed.⁶ This finding contradicts Fesik's original work which determined an enthalpic origin of additivity in the context of FBDD, a finding they attribute to proton transfer events that affect the catalytic domain of stromelysin-1 (SCD) but not MMP12. Here, we revisit the original Fesik ligand series making structural modifications to ensure that proton transfer events are fully accounted for in the additivity analysis.

In their original report, Fesik and coworkers analyzed a series of linkage ligands for stromelysin-1 based on biphenol and hydroxamic acid fragments (Fig. 1).¹ While phenols are attractive from an experimental perspective, primarily because of increased aqueous solubility relative to corresponding ether, they are both hydrogen bond donors and acceptors, while ethers act only as hydrogen bond

* Corresponding author. Tel.: +1 919 681 3484; fax: +1 919 660 0000.

E-mail address: eric.toone@duke.edu (E.J. Toone).

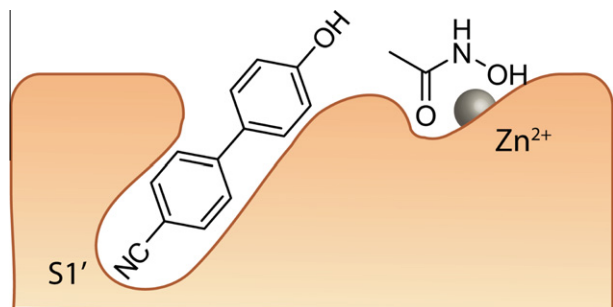


Figure 1. Stromelysin-1 binding biphenol moiety in the S1' pocket and acetohydroxamic acid chelating catalytic zinc.

acceptors. Additionally, no correction was made for proton transfer events commonly observed for MMPs⁷ and certainly plausible for electron deficient phenols.

The conservation of atoms and functional groups is of paramount importance during linkage ligand design, as the tether itself can interact—favorably or unfavorably—with the protein. Such issues were considered when designing the ligand series depicted in Figure 2. Linkage ligands **1–4**, and acetohydroxamic acid **8** were previously studied by Fesik. Potentially important differences between phenols and phenyl ethers led to the inclusion of compounds **5–7**, which are ether equivalents to the phenols previously studied by Fesik. To ensure conservation of atoms, hydroxamic acid fragments **9** and **10** were extended to account for all atoms of the linkage ligands.

Synthesis of all ligands proceeded uneventfully.⁸ Linkage ligands **1–4** were synthesized according to previously reported procedures.⁹ Ligand fragments **5–7** were made by reacting the corresponding phenol with an iodoalkane.¹⁰ Hydroxamic acids **8–10** were made by reacting the corresponding ester with hydroxylamine under methanolic conditions.¹¹ Recombinant stromelysin-1 catalytic domain (SCD) was expressed in *Escherichia coli* according to previously described methods¹² and dialyzed into

calorimetry buffer containing 50 mM buffer (Tris–HCl, HEPES or MOPS), pH 7.5, 10 mM CaCl₂, 1 μM ZnCl₂. Protein concentrations were determined by the method of Edelhoch.¹³

Table 1 summarizes the observed thermodynamics of binding. Three percent DMSO was added to all solutions to facilitate complete dissolution. The binding of compounds **1** and **4** was characterized by direct titration ITC; the weakly binding linkage ligands **2** and **3** and fragments **8–10** were characterized via displacement calorimetry.¹⁴ The hydrophobic fragments (compounds **5–7**) presented challenges due to poor solubility and only partial displacement titrations were conducted.¹⁵ Binding thermodynamics of all ligands were characterized in both Tris–HCl and HEPES; if differences in enthalpy were observed in the two buffers, a third titration in MOPS was conducted to determine the extent of proton transfer by linear regression; ΔH_{bind} values reported in Table 1 are corrected for proton transfer. All experiments were conducted in triplicate in Tris–HCl and in duplicate for MOPS and HEPES buffers. The errors reported ΔG° , ΔH° , represent the standard deviation between experiments. The errors for $T\Delta S$ and ΔJ_i were determined via prorogation of errors; those errors for ΔC_p were determined by regression. With the exception of compound **6**, titrations were conducted at 15, 25 and 37 °C to determine change in molar heat capacity (ΔC_p) accompanying binding. Compound **6** was insoluble below room temperature, precluding ΔC_p determination.

Proton transfer events are common among MMPs and previously reported for stromelysin-1.^{7,16,17} The original characterization of the biphenyl ligands did not include proton transfer events and was conducted in Tris–HCl, a buffer which has an exceptionally large ionization enthalpy ($\Delta H_{\text{ion}} = -11.45 \text{ kcal mol}^{-1}$). Using the equation

$$\Delta H_{\text{bind}} = \Delta H_{\text{obs}} + n\Delta H_{\text{ion}}$$

to account for proton transfer where n is the number of protons transferred upon binding (0.21) and ΔH_{obs} is the observed enthalpy, our results are in good agreement with those of Fesik (Supplemental materials). The difference in enthalpy for the phenol ($\Delta H_{\text{obs}} = -4.7 \text{ kcal mol}^{-1}$ in Tris–HCl) and ether fragment **5** ($\Delta H_{\text{bind}} = -1.6 \text{ kcal mol}^{-1}$) would correspond to an n of

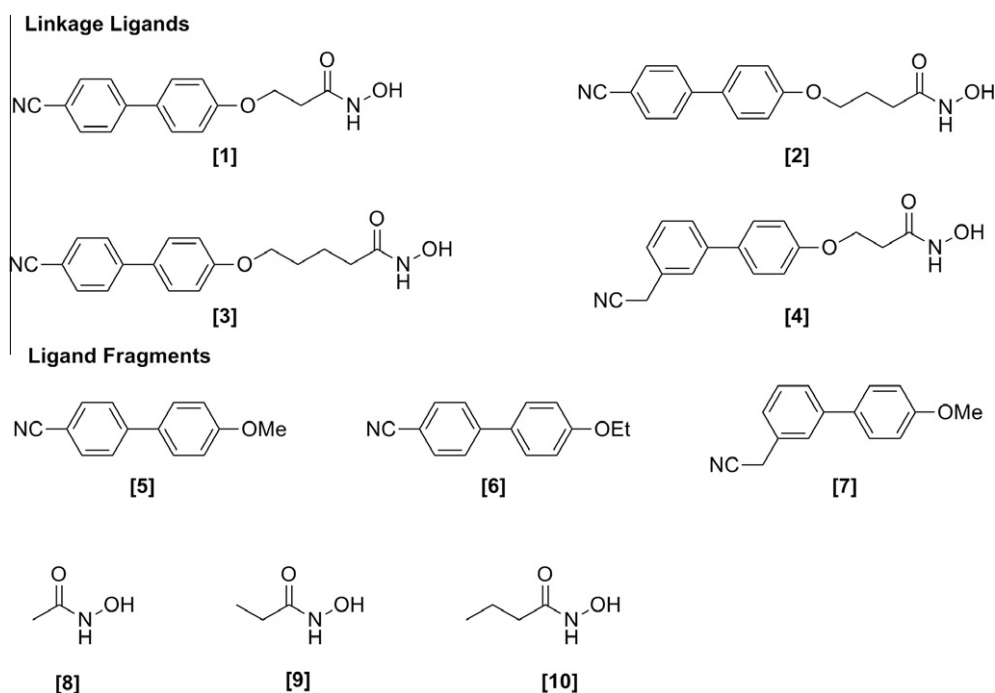


Figure 2. Modified biphenyl series of ligands.

Table 1

Thermodynamics of ligand binding for all compounds determined by isothermal titration calorimetry, corrected for proton transfer events

Compound	ΔG^0	ΔH_{bind}^0	$T\Delta S_{\text{bind}}^0$	ΔC_p
1	-9.5 ± 0.5	-11.5 ± 0.2	-2.0 ± 0.5	-72 ± 2
2	-6.4 ± 0.1	-8.0 ± 0.4	-1.6 ± 0.4	-60 ± 11
3	-6.6 ± 0.2	-7.5 ± 0.2	-0.9 ± 0.2	-67 ± 16
4	-10.5 ± 0.2	-9.2 ± 0.4	-0.8 ± 0.4	-59 ± 5
5	-4.8 ± 0.1	-1.6 ± 0.3	3.2 ± 0.3	-48 ± 60
6	-5.3 ± 0.1	-0.8 ± 1.0	4.5 ± 1.0	nd
7	-6.1 ± 0.4	-1.5 ± 0.3	4.6 ± 0.4	-41 ± 6
8	-2.4 ± 0.3	-2.3 ± 0.2	0.1 ± 0.3	-67 ± 24
9	-2.6 ± 0.1	-3.3 ± 0.4	-0.7 ± 0.4	-54 ± 8
10	-2.7 ± 0.1	-2.2 ± 0.1	0.5 ± 0.1	-59 ± 6

ΔG , ΔH_{bind} , $T\Delta S$ reported in kcal mol⁻¹.

ΔC_p reported in cal mol⁻¹ K⁻¹.

0.27. 4-Cyanophenol has pK_a of 7.95.¹⁸ Addition of a second phenyl ring should further decrease the pK_a making it plausible that the phenol fragment is partially deprotonated at pH 7.5.

Using the data of Table 1, an additivity analysis was conducted according to the method of Jencks (Table 2). In the cases of compounds **2** and **3**, two different combinations of ligand fragments form the linkage ligands, and both combinations were analyzed. Additionally, the linking coefficient, *E*, is also reported for each linkage ligand and representative ligand fragment pairing. This construct, recently described by Whittaker and coworkers describes the efficiency of fragment linkage. The linking coefficient, *E*, is related to the dissociation constant *K_D* such that

$$K_D^{AB} = K_D^A K_D^B E$$

or the ΔG_i such that

$$\Delta G_i = -RT \ln E$$

Values less than 1 demonstrate a favorable thermodynamic consequence of ligand linkage, values near 1 indicate linkage was thermodynamically neutral, and values greater than 1 indicate a negative thermodynamic consequence of fragment linkage.¹⁹

Several features of the Table 2 data are noteworthy. First, little variation is observed in $T\Delta S_i$ across the entire series, with an average of 4.7 ± 1.0 kcal mol⁻¹. The consistency of this value suggests that ΔS_i is a property of the protein, rather the ligand. The variability among ΔH_i is larger than ΔS_i ; the average interaction enthalpy is 4.7 ± 1.7 kcal mol⁻¹. The discrepancy between the ΔH_i for compounds **1/4** versus **2/3** has been previously attributed to the ability of compounds **1** and **4** to hydrogen bond with Leu164. This hydrogen bond interaction is absent upon tether elongation.¹

Examination of Table 2 reveals the counterintuitive result that favorable additivity in ligand binding—that is a free energy of binding greater than the sum of those for the constituent ligand fragments—is *enthalpic* in origin. The most frequently cited

conceptual underpinning for FBDD involves a ‘savings’ in translational and rotational entropy achieved by fragment tethering, which effectively converts two particles to one, and diminishes the loss in translational and rotational entropy by one particle. Our results stand in direct opposition to this hypothesis. In all cases, ligand linkage is associated with a significant enthalpic benefit and large entropic penalty. Favorability of ligand linkage is determined solely by the magnitude of ΔH_i . Consistent with this notion is the greater variation in enthalpy rather than entropy.

One of two constructs is typically invoked to rationalize enthalpic additivity: hydrophobic effects^{20–22} and interfacial mobility/protein contraction.^{21,23} Hydrophobic interactions have both an enthalpic and entropic component. Van der Waals forces contribute to the association enthalpy as attractive forces between hydrophobic moieties are more favorable than the interactions of a hydrophobic moiety and water. The classical hydrophobic effect argues that entropic effects dominate hydrophobic interactions,^{20–22,24–26} while a non-classical view hydrophobic effects focuses on enthalpic contributions.^{27–31}

The enthalpic component of the hydrophobic effect arises primarily from van der Waals forces, and the magnitude of this effect trends with ligand surface area.^{32,33} Thus, linkage ligands with increased tethers should show increased binding enthalpies, assuming an increased surface area—that of the tether—is removed from solvent during binding. If the enthalpy of binding decreases for a ligand of equal or greater surface area, then the loss in enthalpy results from some other effect, for example, the loss of a hydrogen bond, an unfavorable ligand conformation, or trapping of a lone pair along the hydrophobic interface.²¹ Our experimental results demonstrated a decreased enthalpy of binding upon tether elongation, while Fesik and co-workers previously reported that tether elongation resulted in the loss of a hydrogen bond.^{1,9}

Just as elongation of an alkyl tether should increase the enthalpic benefit of binding, the entropy of binding should also increase with increasing ligand size. While desolvated ligand surface areas were not calculated here, binding entropy should increase in a linear manner as methylene units are added to the linker. Rather, we observe remarkable consistency in both the entropies of binding and the interaction entropies observed with the biphenyl linkage ligands. No increase in binding entropy was observed with tether elongation as predicted by a notion of hydrophobicity-driven binding.

Finally, hydrophobic hydration affects ΔC_p in a predictable fashion. While changes in electrostatics or vibrational states of a protein during binding could, in principle, impact ΔC_p ,³⁴ ΔC_p is the best available measurement of changes in solvent-exposed hydrophobic surface area and is therefore considered a hallmark of the hydrophobic effect.^{35–37} The values of ΔC_p observed here offer no evidence for major differences in the solvation behavior of the protein during binding. While there have been recent reports of Grb-2 SH2 domain exhibiting hydrophobic binding in the absence of changes in ΔC_p , the enthalpic and entropic trends of ligand binding were as predicted by the hydrophobic effect. In this case, neither ΔH or $T\Delta S$ nor ΔC_p behave in a manner consistent with the hydrophobic effect and we therefore reject this rationale for ligand binding.

Protein contraction and the interfacial mobility model arises from a tightening of the ligand–protein interface that maximizes enthalpic intermolecular forces (ionic, van der Waals, and dipole–dipole interactions), which vary inversely with intermolecular distance.^{38–40} These favorable enthalpic interactions are, in part, offset by entropic penalties that arise from protein contraction with resultant rigidification. As ligand size increases (as measured by the number of protein residues contacted), the interfacial tightening required to maximize enthalpic interactions requires too high an entropic penalty and enthalpy

Table 2

Additivity analysis of biphenyl ligands. Thermodynamic parameters reported in kcal mol⁻¹

Ligand	Ligand fragments		ΔG_i^0	ΔH_i^0	$T\Delta S_i^0$	<i>E</i>
	S1'	Zn2+				
1	5	8	-2.3 ± 0.6	-7.6 ± 0.6	-5.3 ± 0.7	0.021
	2	9	1.0 ± 0.3	-3.1 ± 0.6	-4.1 ± 0.6	5.354
2	6	8	1.3 ± 0.4	-4.9 ± 1.1	-6.2 ± 1.1	8.857
	3	10	0.9 ± 0.4	-3.7 ± 0.4	-4.6 ± 0.4	4.527
3	6	9	1.3 ± 0.4	-3.4 ± 1.1	-4.7 ± 1.1	8.857
	4	7	-2.0 ± 0.7	-5.4 ± 0.5	-3.4 ± 0.8	0.035

ΔG , ΔH_{bind} , $T\Delta S$ reported in kcal mol⁻¹.

ΔC_p reported in cal mol⁻¹ K⁻¹.

falls short of the maximum available. A diminished tightening produces less rigidification (and a diminished entropic penalty relative to that which would accompany maximum enthalpic interaction). From the perspective of free energy, as ligand size increases, diminished favorable enthalpy (due to failure to maximize interactions) is compensated by a diminished entropic penalty (less rigidification). Unfortunately, the linkage ligands described here all contact the same protein residues.¹ Evidence supported this theory include the consistency of ΔS among linkage ligands and the lack of change in ΔC_p as they hydrophobic effect would not be expected to contribute. Further, we have recently demonstrated that trends in enthalpy–entropy compensation do appear to trend with protein contraction for SCD.⁴¹ To fully probe whether interfacial mobility and protein contraction could explain the trends seen here, a ligand series contacting more regions of the SCD protein would be required.

At least for the case of stromelysin-1, FBDD appears to be enthalpically favored and entropically opposed. The origin of the discrepancy between our results and those recently published by Borsi et al.⁶ is unclear. One possibility is proton transfer events. Consideration of both ligand binding and proton transfer events is critical to interpretation of thermodynamic parameters. Many functional groups have pK_a s in the physiologic range (pH 6–8), including sulfonamides^{42–44} and phenols,^{45,46} highlighting the importance of including proton transfer experiments during any thermodynamic characterization. Clearly additional work is required to ascertain whether the results reported here are generalizable, and we will report our results in due course.

Acknowledgments

E.J.T. acknowledges funding for this work by the NIH (1R01GM57179). E.M.W. acknowledges funding for her training from the NIH (5T32GM007171).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2012.05.032>.

References and notes

- Olejniczak, E. T.; Hajduk, P. J.; Marcotte, P. A.; Nettesheim, D. G.; Meadows, R. P.; Edalji, R.; Holzman, T. F.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 5828.
- Hajduk, P. J.; Greer, J. *Nature Reviews Drug Discovery* **2007**, *6*, 211.
- Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4046.
- Ahmad, N.; Gabius, H.-J.; Sabesan, S.; Oscarson, S.; Brewer, C. F. *Glycobiology* **2004**, *14*, 817.
- Lundquist, J. J.; Debenham, S. D.; Toone, E. J. *J. Org. Chem.* **2000**, *65*, 8245.
- Borsi, V.; Calderone, V.; Fragai, M.; Luchinat, C.; Sarti, N. *J. Med. Chem.* **2010**, *53*, 4285.
- Parker, M. H.; Lunney, E. A.; Ortwin, D. F.; Pavlovsky, A. G.; Humblet, C.; Brouillette, C. G. *Biochemistry* **1999**, *38*, 13592.
- Du, Y., Duke University 2006.
- Hajduk, P. J.; Sheppard, G.; Nettesheim, D. G.; Olejniczak, E. T.; Shuker, S. B.; Meadows, R. P.; Steinman, D. H.; Carrera, G. M.; Marcotte, P. A.; Severin, J.; Walter, K.; Smith, H.; Gubbins, E.; Simmer, R.; Holzman, T. F.; Morgan, D. W.; Davidsen, S. K.; Summers, J. B.; Fesik, S. W. *J. Am. Chem. Soc.* **1997**, *119*, 5818.
- Civitello, E. R.; Rapoport, H. J. *Org. Chem.* **1994**, *59*, 3775.
- Reddy, A. S.; Kumar, M. S.; Reddy, G. R. *Tetrahedron Lett.* **2000**, *41*, 6285.
- Wilfong, E. M.; Locklear, U. N.; Toone, E. J. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 280.
- Edelholz, H. *Biochemistry* **1948**, *1967*, 6.
- Sigurskjold, B. W. *Analytical Biochemistry* **2000**, *277*, 260.
- Christensen, T.; Gooden, D. M.; Kung, J. E.; Toone, E. J. *J. Am. Chem. Soc.* **2003**, *125*, 7357.
- Holman, C. M.; Kan, C.-C.; Gehring, M. R.; Van Wart, H. E. *Biochemistry* **1999**, *38*, 677.
- Johnson, L. L.; Pavlovsky, A. G.; Johnson, A. R.; Janowicz, J. A.; Man, C. F.; Ortwin, D. F.; Purchase, C. F.; White, A. D.; Hupe, D. J. *J. Biol. Chem.* **2000**, *275*, 11026.
- Wheland, G. W.; Brownell, R. M.; Mayo, E. C. *J. Am. Chem. Soc.* **1948**, *70*, 2492.
- Ichihara, O.; Barker, J.; Law, R. J.; Whittaker, M. *Molecular Informatics* **2011**, *30*, 298.
- Hookey, R. J.; Van Anda, H. J.; Rebek, J. J. *J. Am. Chem. Soc.* **2007**, *129*, 13464.
- Krishnamurthy, V. M.; Bohall, B. R.; Semetey, V.; Whitesides, G. M. *J. Am. Chem. Soc.* **2006**, *128*, 5802.
- Tanford, C. *Science* **1978**, *1012*, 200.
- Williams, D. H.; Stephens, E.; O'Brien, D. P.; Zhou, M. *Angew. Chem. Int. Ed.* **2004**, *43*, 6596.
- Schote, U.; Ganz, P.; Fahr, A.; Seelig, J. *J. Pharm. Sci.* **2002**, *91*, 856.
- Schote, U.; Seelig, J. *Biochim. Biophys. Acta Biomembr.* **1998**, *1415*, 135.
- Tanford, C. *The Hydrophobic Effect*; John Wiley & Sons: New York, 1980.
- Ackroyd, P. C.; Cleary, J.; Glick, G. D. *Biochemistry* **2001**, *40*, 2911.
- Cole, J. L.; Garsky, V. M. *Biochemistry* **2001**, *40*, 5633.
- Malham, R.; Johnstone, S.; Bingham, R. J.; Barratt, E.; Phillips, S. E. V.; Laughton, C. A.; Homans, S. W. *J. Am. Chem. Soc.* **2005**, *127*, 17061.
- Seelig, J.; Ganz, P. *Biochemistry* **1991**, *30*, 9354.
- Syme, N. R.; Simon, C. D.; Phillips, E. V.; Homans, S. W. *ChemBioChem* **2007**, *8*, 1509.
- Chandler, D. Oxford University Press, New York, 1987, pp 5.
- Chandler, D. *Nature* **2005**, *437*, 640.
- Sturtevant, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 2236.
- Spolar, R. S.; Record, M. T. *Science* **1994**, *263*, 777.
- Murphy, K. P.; Freire, E. *Adv. Protein Chem.* **1992**, *43*, 313.
- Livingstone, J. R.; Spolar, R. S.; Record, M. T. *Biochemistry* **1991**, *30*, 4237.
- Hornig, J. F.; Hirschfelder, J. O. *J. Chem. Phys.* **1912**, *1952*, 20.
- London, F. Z. *Phys.* **1930**, *63*, 245.
- Margenau, H. J. *Chem. Phys.* **1938**, *6*, 896.
- Wilfong, E. M.; Kogiso, Y.; Muthukrishnan, S.; Kowatz, T.; Du, Y.; Bowie, A.; Naismith, J. H.; Hadad, C. M.; Toone, E. J.; Gustafson, T. L. *J. Am. Chem. Soc.* **2011**, *133*, 11515.
- Cammarata, A.; Allen, R. C. *J. Pharm. Sci.* **1967**, *56*, 640.
- Remko, M.; von der Lieth, C.-W. *Bioorg. Med. Chem.* **2004**, *12*, 5395.
- Combinatorial Chemistry*; Simon, M. I., Abelson, J. N., Eds.; Academic Press: San Diego, 1996.
- Fujita, T. *J. Med. Chem.* **1966**, *9*, 797.
- Liptak, M. D.; Gross, K. C.; Seybold, P. G.; Feldgus, S.; Shields, G. C. *J. Am. Chem. Soc.* **2002**, *124*, 6421.