Theoretical and Experimental Studies of Biotin Analogues That Bind Almost as Tightly to Streptavidin as Biotin

Richard W. Dixon,* Randall J. Radmer, Bernd Kuhn, and Peter A. Kollman

Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California 94143

Jaemoon Yang, Cesar Raposo, and Craig S. Wilcox

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Lisa A. Klumb and Patrick S. Stayton

Department of Bioengineering, University of Washington, Seattle, Washington 98195

Craig Behnke, Isolde Le Trong, and Ronald Stenkamp

Department of Biological Structure, University of Washington, Seattle, Washington 98195

richard.dixon@abbott.com

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We have used a newly developed qualitative computational approach, PROFEC (Pictorial Representation of Free Energy Changes), to visualize the areas of the ligand biotin where modifications of its structure might lead to tighter binding to the protein streptavidin. The PROFEC analysis, which includes protein flexibility and ligand solvation/desolvation, led to the suggestion that the *pro*-9*R* hydrogen atom of biotin, which is in α-position to the CO_2^- group, might be changed to a larger group and lead to better binding with streptavidin and avidin. Free energy calculations supported this suggestion and predicted that the methyl analogue should bind ≈3 kcal/mol more tightly to streptavidin, with this difference coming exclusively from the relative desolvation free energy of the ligand. The PROFEC analysis further suggested little or no improvement for changing the *pro*-9*S* hydrogen atom to a methyl group, and great reduction in changing the ureido N−H groups to N−CH_3. Stimulated by these results, we synthesized 9*R*-methylbiotin and 9*S*-methylbiotin, and their binding free energies and enthalpies were measured for interaction with streptavidin and avidin, respectively. In contrast to the calculated results, experiments found both 9-methylbiotin isomers to bind more weakly to streptavidin than biotin. The calculated preference for the binding of the 9*R*- over the 9*S*-stereoisomer was observed. In addition, 9-methylbiotin is considerably less soluble in water than biotin, as predicted by the calculation, and the 9*R* isomer is, to our knowledge, thus far the tightest binding analogue of biotin to streptavidin. Subsequently, X-ray structures of the complexes between streptavidin and both 9*R*- and 9*S*-methylbiotin were determined, and the structures were consistent with those used in the free energy calculations. Thus, the reason for the discrepancy between the calculated and experimental binding free energy does not lie in unusual binding modes for the 9-methylbiotins.

Introduction

Given a structure of a macromolecule, the goal of structure-based design is to find ligands that bind to it tightly and specifically. The computational methodologies designed to do so vary widely,[1] from methods that analyze databases with many thousands of ligands,[2] to methods that focus on single, small changes such as free energy perturbation calculations.[3] Whereas the simplest methods must use very approximate scoring schemes to filter good from bad candidate ligands, free energy approaches can, in principle, consider such critical aspects as ligand solvation/desolvation and (limited) binding site flexibility. The difficulty with a free energy calculation is its tremendous computational demand. That is why one of us (R.J.R.) has developed a more approximate method, PROFEC (Pictorial Representation Of Free Energy Changes), for ligand design that can use modest length molecular dynamics trajectories on the solvated ligand and the solvated ligand−macromolecule complex to estimate where a "lead" compound can best be modified to bind more tightly to the macromolecule. This method looks quite promising, having qualitatively ranked the observed relative free energies of binding of benzamidine analogues to trypsin.[4]

We decided to try PROFEC on a more challenging problem such as the biotin−streptavidin system. The complexes of biotin with streptavidin and its structural homologue avidin are known to be among the strongest ligand-protein complexes,[5−10] with measured binding

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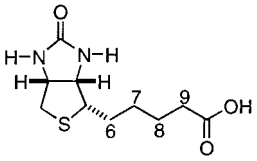
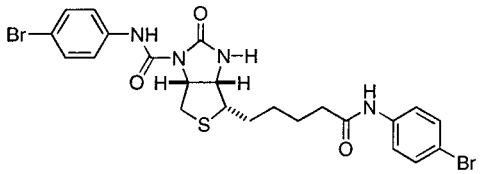
constants of 1.7×10^{15} and 2.5×10^{13}, respectively.[5−8,11] Because of the very high binding affinities of biotin to these two proteins, which are certainly the highest known per number of atoms in the ligand (excluding metal complexes), many studies have been directed to understand and elucidate the mechanism of binding at the molecular level.[9,10,12,13] Given this strong binding, it is a significant challenge to predict new biotin analogues that bind even more tightly to streptavidin than biotin itself.

The reference ligand D-(+)-biotin **1**, vitamin H, serves as a CO_2 carrier[14] and was first isolated as a growth factor for yeast in 1936 in its methyl ester form.[15]



1

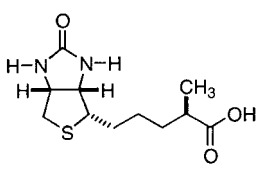
The absolute configuration of D-(+)-biotin was determined by measurement of the anomalous dispersion of X-ray data on bis-*p*-bromoanilide carbon dioxide biotin **2**.[16] Subsequently, X-ray crystallographic data on biotin itself, and its analogues have been published.[17]



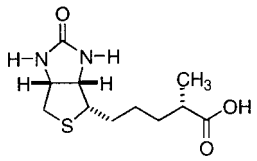
2

The proposed structure for the D-(+)-biotin was confirmed by the first total synthesis by Merck[18] and later numerous organic syntheses employing different methodologies have appeared.[19]

9*R*- and 9*S*-methylbiotin, **3a**/**3b**, are structural analogues of D-(+)-biotin, first isolated in 1971 from the fermentation of a strain of *S. lydicus* on *Saccharomyces pastorianus*.[20] Studies of 9-methylbiotin on the antimicrobial activity were performed in detail to show that it could not substitute for the biotin requirement for the growth of *Saccharomyces cerevisiae* although it has affinity for avidin.[21]

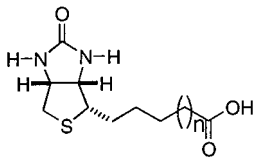


3a



3b

Unlike other biotin homologues such as (+)-norbiotin (**4a**), (+)-homobiotin (**4b**), (+)-bis-homobiotin (**4c**), and (+)-tris-homobiotin (**4d**), 9-methylbiotin keeps the valeric acid side chain and has an additional methyl substituent in α-position to the carboxylic acid group.



4a, n=0
4b, n=2
4c, n=3
4d, n=4

Streptavidin and avidin have very similar primary structures and can be isolated from the bacterium *Streptomyces avidinii* and from hen egg white, respectively. They are both tetrameric proteins, but differ in their overall charge, with streptavidin being highly acidic and avidin being highly basic. X-ray structures of biotin cocrystallized with both streptavidin[22−24] and avidin[13] revealed the protein residues involved in binding.

In the following, we describe a combined theoretical and experimental approach to investigate the binding affinities of different methylbiotin analogues to streptavidin and avidin. This involves the initial PROFEC analysis, subsequent binding free energy and solvation free energy calculations, organic synthesis of the two 9-methylbiotin stereoisomers, thermodynamic measurements of the binding affinities of the 9-methylbiotins to streptavidin and avidin, and X-ray analysis of the binding modes of these compounds in streptavidin.

Methods

A. Theoretical Calculations. The quantitative determination of free energy changes is perhaps the most powerful tool available to the computational chemist. In principle, given two states A and B, the free energy associated with converting the system in question from A to B can be calculated by

$$\Delta G = G_B - G_A = -RT \ln\langle e^{[V_B(x) - V_A(x)]/RT}\rangle_A \qquad (1)$$

In this case, sampling of configuration space is performed at state A according to a potential energy function V_A. The accuracy of the calculated ΔG in eq 1 is determined by the quality of the potential function V_A and the completeness of the sampling of configuration space during the course of the simulation. In practice, it has been found that the highest quality results can be obtained when states A and B are

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similar. Therefore, a number of innovations have become common practice in the determination of free energy changes through simulation methodology. The first is that the path between states A and B is usually divided into a number of discrete steps, with a coupling parameter λ defining this path, where $\lambda = 0$ at A and $\lambda = 1$ at B. This allows a conversion between two states to be represented as a collection of arbitrarily small subdivisions. The free energy change at any point along the simulation path is defined as

$$\Delta G_{\lambda_i} = -RT \ln\langle e^{[V_{\lambda_{i+1}}(x) - V_{\lambda_i}(x)]RT}\rangle_{\lambda_i} \quad (2)$$

The method described in eqs 1 and 2 is called free energy perturbation. An alternative way to calculate the free energy by slowly varying the Hamiltonian with the coupling parameter λ is called thermodynamic integration (eq 3).

$$\Delta G = \int_0^1 \left\langle \frac{\partial H}{\partial \lambda} \right\rangle_\lambda d\lambda \quad (3)$$

Both of these methods have been extensively used in free energy calculations.[3]

Another conceptual change made in free energy calculations is to simulate "nonphysical" paths and take advantage of the state function nature of the free energy to relate the results to physical processes. Thus, thermodynamic cycles are invoked, such as the one in eq 4, for the relative binding free energy of two ligands to a protein.

$$\begin{array}{ccc} \text{protein} + \text{ligand}_1 & \xrightarrow{\Delta G_3} & (\text{protein} : \text{ligand}_1) \\ \downarrow \Delta G_1 \equiv \Delta G_{solv} & & \downarrow \Delta G_2 \equiv \Delta G_{prot} \\ \text{protein} + \text{ligand}_2 & \xrightarrow{\Delta G_4} & (\text{protein} : \text{ligand}_2) \end{array}$$

$$\Delta G_{prot} - \Delta G_{solv} = \Delta G_4 - \Delta G_3 = \Delta G_{tot} \quad (4)$$

The free energy calculations, which have been performed as a part of this effort, have all relied on the above methodology. The details of these calculations will be described below.

A major drawback of these calculations is that they carry the price of significant computational burden. If one wishes to examine the effects of a relatively large number of changes between ligand systems, the computational needs may soon outstrip available resources. For this reason, a qualitative methodology has been developed which allows relatively rapid assessment of a broad range of free energy changes. In this approach, called PROFEC (Pictorial Representation Of Free Energy Changes), eq 5, in analogy to eq 1, is used to calculate the free energy cost of inserting a particle at a point space (i,j,k).

$$\Delta G(i,j,k) = -RT \ln\langle e^{-\Delta V(i,j,k)/RT}\rangle \quad (5)$$

$\Delta V(i,j,k)$ is the van der Waals interaction energy between a Lennard-Jones particle and the surrounding atoms. The ensemble average $\langle ...\rangle_A$ is calculated from stored trajectories collected from a single simulation involving the reference ligand, state A, only. This exercise is carried out for a grid of insertion points surrounding the reference ligand. Given a set of grid points, each with an associated van der Waals free energy value, contours can be drawn with a suitable graphics program corresponding to different values of free energy. For example, the contour of 0.0 kcal/mol represents a boundary for free energy changes. Particles that fall "inside" this contour are predicted to lead to improved interaction free energies, while those outside are predicted to have a deleterious effect on the interactions.

The electrostatic interactions can be examined by calculating the derivative of the binding free energy at each grid point once the particle has been inserted. This derivative can also be calculated in the absence of the Lennard-Jones particle; however, it has been found to be more useful to weight the electrostatic calculation with the steric favorability of the proposed change. This derivative is calculated by means of umbrella sampling

$$\left[\frac{dG(i,j,k)}{dq}\right]_{LJ(i,j,k)} = \frac{\left\langle \frac{dV(i,j,k)}{dq} e^{-\Delta V(i,j,k)/RT} \right\rangle_A}{\langle e^{-\Delta V(i,j,k)/RT}\rangle_A} = \frac{\langle \Phi(i,j,k) e^{-\Delta V(i,j,k)/RT}\rangle_A}{\langle e^{-\Delta V(i,j,k)/RT}\rangle_A} \quad (6)$$

displayed on the van der Waals free energy contours as color. The resulting image can be used to identify regions of the ligand susceptible to derivatization and to predict the effect of the charge distribution on the proposed physical changes. Once a broad scan of the free energy "surface" has been completed, and candidates chosen, the more CPU-intensive free energy simulation techniques can be used to evaluate each candidate individually. This prescription has been followed for the ligand biotin in the protein streptavidin.

The trajectory information necessary to calculate the free energies needed for PROFEC was collected from a 500 ps simulation of biotin in tetrameric streptavidin using the Cornell et al. force field.[25] An 18 Å belly of 231 TIP3P[26] water molecules was centered on one biotin in the system, and a small harmonic restraint force was applied to the backbone atoms of the mobile portions of the protein. This last restraint was found to play an important role in the free energy simulations and so was included in these calculations for consistency. In addition, SHAKE[27,28] was applied to all bonds, and an 8.0 Å cutoff for nonbonded interactions was imposed. The system temperature was maintained at 300 K by means of the Berendsen temperature coupling algorithm. All simulations began with at least 40 ps of equilibration. Trajectory information was collected every 0.5 ps, resulting in 1000 coordinate sets, which were used to calculate the appropriate ensemble averages. A second simulation of biotin solvated in a box of 555 TIP3P water molecules, approximately 25 × 25 × 30 Å^3, was performed with the same total time and trajectory collection history. A 0.5 Å grid was placed around the biotin molecule, and a united atom carbon atom ($R^* = 2.00$ Å, $\epsilon = 0.15$ kcal/mol) was inserted at each grid point. A series of grids, each requiring a separate PROFEC calculation, was chosen due to the flexible nature of the ligand.

The free energy simulations were carried out in both the forward and reverse direction between biotin and the biotin analogues reported in the next section. Simulation conditions were the same as those described above. The simulation time for each leg of the calculation was 200 ps with a time step of 1 fs. Two hundred windows, each composed of 500 steps of equilibration and 500 steps of data collection, were employed in total. Twenty ps of equilibration was performed prior to each reverse simulation. The reported errors in the calculated free energy values represent the difference between the forward and reverse simulations. Thermodynamic integration[29] was used to calculate the free energy changes.

The atomic partial charges for biotin were those used previously[9,10] and were calculated by means of the RESP methodology[30] at the HF/6-31G* level[31] of theory. Charges for

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the methylbiotin analogues were obtained by using partial charges from a calculation on methane for the methyl group and adjusting the charge of the attachment point atom to maintain correct overall charge.

We also carried out MM-PBSA (molecular mechanics Poisson Boltzmann Surface Area) binding free energy calculations[32] on biotin and its 9*R*- and 9*S*-methyl analogues. We first used the crystal structure of the biotin−streptavidin complex to model the methyl analogues; subsequently, we used the experimental X-ray structures of the 9-methylbiotin−streptavidin complexes. The method of analyzing the binding free energies is described in ref 32.

B. Experimental Data for Synthetic Work. Biotin Methyl Ester (7). A mixture composed of d-(+)-biotin **1** (2.90 g, 11.9 mmol), MeOH (60 mL), and sulfuric acid (6 g) was refluxed under N_2 for 5 h, and then 10 mL of MeOH was removed by distillation. Heating at reflux was continued for an additional 12 h, and then all MeOH was removed by rotary evaporation. The residue was added to a mixture of ice and water, and the pH of the resulting solution was adjusted (final pH ∼ 6) with 25% NaOH. The precipitated solid was extracted with EtOAc and washed with brine. The organic layer was dried ($MgSO_4$) and filtered. The filtrate was concentrated in vacuo to give d-(+)-biotin methyl ester **7** (2.31 g, 75%) as a white solid: $[\alpha]^{23}_D$ +52 (*c* 0.21, $CHCl_3$) ([28] $[\alpha]^{22}_D$ +57 (*c* 1, $CHCl_3$)); R_f 0.24 (5% MeOH−CH_2Cl_2); mp 160−161 °C; IR (KBr) 3252, 2913, 1734, 1698, 1462 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.24 (br s, 1 H), 4.91 (br s, 1 H), 4.53 (m, 1 H), 4.34 (m, 1 H), 3.68 (s, 3 H), 3.17 (m, 1 H), 2.94 (dd, J = 12.8, 5.1 Hz, 1 H), 2.75 (d, J = 12.8 Hz, 1 H), 2.35 (t, J = 7.3 Hz, 2 H), 1.80−1.40 (m, 6 H); HRMS calcd for $C_{11}H_{18}N_2O_3S$ 258.1038, found 258.1048.

[9*R* and 9*S*]-Methylbiotin Methyl Ester (8a). To THF (75 mL) under an argon atmosphere at −78 °C were added 40 mL (20 mmol) of a 0.5 M solution of KHDMS in toluene (dropwise, over 5 min) and 3.0 mL (20 mmol) of TMEDA. The mixture was stirred for an additional 5 min, and then a solution of (+)-biotin methyl ester **7** (1.29 g, 5 mmol) in a mixture of HMPA/THF (10 mL/7.5 mL) was added via syringe over 25 min. Stirring was continued for 1 h at −78 °C, the color of the solution changed to light brown, and then 2.50 mL of MeI (40 mmol) was added dropwise. The reaction mixture was stirred for 1.5 h at −78 °C, 25 mL of 10% aqueous HCl was added to the cold mixture, cooling was discontinued, and the mixture was warmed to 25 °C. Volatile components were removed by rotary evaporation, and the product was extracted with CH_2Cl_2 (10 × 20 mL), washed with brine (1 × 20 mL), and dried ($MgSO_4$). After filtration, the filtrate was concentrated, and residual HMPA was removed by Kugelrohr vacuum distillation. Purification of the residue by flash chromatography (SiO_2, 5% MeOH−CH_2Cl_2) afforded 1.95 g of material, which was a mixture (1:6 by ^1H NMR) of starting material and α-methylated product. Further purification was achieved by preparative HPLC using a C18 silica column (10 mm ID × 25 cm) and 0.5% aqueous TFA:CH_3CN (75:25 v:v) at a flow rate of 2 mL/min. (The pH of the TFA solution was adjusted to 2.5−3.0 by using 33% KOH.)

In this manner we obtained 0.88 g (64%) of pure 9*R*- and 9*S*-methylbiotin methyl ester **8a**: $[\alpha]^{23}_D$ +39 (*c* 0.19, $CHCl_3$); R_f 0.24 (5% MeOH−CH_2Cl_2); mp 116−117 °C; IR (KBr) 3245, 2926, 1725, 1698, 1462 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.85 and 4.79 (br m, 1 H), 4.68 (br m, 1 H), 4.54 (m, 1 H), 4.34 (m, 1 H), 3.68 (s, 3 H), 3.17 (m, 1 H), 2.95 (dd, J = 12.7, 5.0 Hz, 1 H), 2.74 (d, J = 12.7 Hz, 1 H), 2.49 (m, 1 H), 1.80−1.30 (m, 6 H), 1.17 (t, J = 7.0 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$ + CD_3OD) δ 177.41, 177.35, 164.05, 61.86, 61.76, 59.92, 55.48, 55.32, 51.50, 40.22, 39.15, 39.00, 33.36, 33.20, 28.31, 28.06, 26.70, 26.41, 16.95, 16.79; HRMS calcd for $C_{12}H_{20}N_2O_3S$ 272.1195, found 272.1191.

[9*R* and 9*S*]-Methylbiotin (3a and 3b). To an ice cold mixture of water (4 g) and TFA (12 g) was added 0.6801 g (2.50 mmol) of (9*R*- and 9*S*-)methylbiotin methyl ester **8a**. The resulting solution was heated at 95−100 °C for 12 h. The mixture was cooled, and volatile components were removed by rotary evaporation. The residual solid was recrystallized from 20 mL of water. After 12 h, the crystalline solid was filtered, washed with water, and vacuum-dried at 65−70 °C for 12 h to afford 0.3992 g (62%) of **3a** and **3b**, (9*R* and 9*S*)-methylbiotin: $[\alpha]^{23}_D$ +109 (*c* 0.010, pH = 9 NH_4OAc buffer); mp 185−188 °C. This product was not further characterized but was immediately converted to the amides.

[9*R* and 9*S*]-Methylbiotin (*N*-(*S*-α-phenethyl))amides (9a and 9b). To an ice-cooled mixture of the (9*R* and 9*S*)-methylbiotin carboxylic acids **3a/3b** (258 mg, 1.0 mmol), CH_2Cl_2 (10 mL), and HMPA (2.5 mL), under argon, was added 486 mg of BOP reagent (1.1 mmol) followed by 0.42 mL (3.3 mmol) of 9*S*-methylbenzylamine. After 10 min, Et_3N (0.14 mL, 1.0 mmol) was added, and the reaction mixture was stirred for 3.5 h. Water (20 mL) was added, the organic phase was separated, and the aqueous layer was extracted with (6 × 2 mL, 5% MeOH−CH_2Cl_2). The combined organic layers were washed with brine and dried ($MgSO_4$). After filtration, the filtrate was concentrated, and residual HMPA was removed by Kugelrohr vacuum distillation. Purification of the residue by flash chromatography (SiO_2, 5% MeOH−CH_2Cl_2) afforded 0.37 g of material which was a 1:1 mixture of two diastereomers as shown by ^1H NMR and by HPLC. Separation of these stereoisomers was achieved by preparative HPLC using a C18 silica column (10 mm ID × 25 cm) and 0.5% aqueous TFA:CH_3CN (75:25 v:v) at a flow rate of 2 mL/min. (The pH of the TFA solution was adjusted to 2.5−3.0 by using 33% KOH.)

(9*S*)-Methylbiotin (*N*-(*S*-α-phenethyl))amide (9b): yield 108 mg (60%); $[\alpha]^{23}_D$ +13 (*c* 0.60, MeOH); R_f 0.12 (5% MeOH−CH_2Cl_2); mp 179−181 °C; IR (KBr) 3283, 2923, 1690, 1678, 1630, 1534 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 7.40−7.20 (m, 5 H), 5.00 (q, J = 7.0 Hz, 1 H), 4.45 (dd, J = 7.6, 5.0 Hz, 1 H), 4.16 (dd, J = 7.7, 4.4 Hz, 1 H), 3.08 (m, 3 H), 2.90 (dd, J = 12.8, 4.8 Hz, 1 H), 2.68 (d, J = 12.7 Hz, 1 H), 2.35 (m, 1 H), 1.80−1.20 (m, 9 H), 1.11 (t, J = 6.8 Hz, 3 H); ^{13}C NMR (75 MHz, CD_3OD) δ 178.58, 166.21, 145.62, 129.67, 128.14, 127.32, 63.45, 61.67, 57.04, 41.98, 41.22, 35.34, 29.67, 28.37, 22.45, 18.53; ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.67, 162.70, 145.06, 128.21, 126.47, 125.88, 61.09, 59.15, 55.55, 47.56, 33.92, 28.25, 26.73, 22.51, 17.89; HRMS calcd for $C_{19}H_{27}N_3O_2S$ 361.1824, found 361.1842.

(9*R*)-Methylbiotin (*N*-(*S*-α-phenethyl))amide (9a): yield 157 mg (87%); $[\alpha]^{23}_D$ −30 (*c* 0.73, MeOH); R_f 0.12 (5% MeOH−CH_2Cl_2); mp 209−210 °C; IR (KBr) 3281, 2917, 1694, 1630, 1534 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 7.40−7.20 (m, 5 H), 5.00 (q, J = 7.0 Hz, 1 H), 4.49 (dd, J = 7.6, 4.7 Hz, 1 H), 4.29 (dd, J = 7.8, 4.4 Hz, 1 H), 3.19 (m, 3 H), 2.93 (dd, J = 12.8, 4.9 Hz, 1 H), 2.71 (d, J = 12.7 Hz, 1 H), 2.38 (m, 1 H), 1.80−1.20 (m, 9 H), 1.11 (t, J = 6.9 Hz, 3 H); ^{13}C NMR (75 MHz, CD_3OD) δ 178.51, 166.25, 145.46, 129.63, 128.13, 127.20, 63.58, 61.76, 57.34, 41.88, 41.19, 35.34, 29.99, 28.58, 22.72, 18.77; ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.67, 162.85, 144.97, 128.32, 126.60, 125.95, 61.31, 59.28, 55.72, 47.53, 33.81, 28.47, 26.80, 22.74, 18.18; HRMS calcd for $C_{19}H_{27}N_3O_2S$ 361.1824, found 361.1836.

(9*R*)-Methylbiotin (3a). A mixture of 1 mL of 48% HBr and amide **9a** (20.0 mg, 55 μmol) was heated at100−105 °C (oil bath) for 3 h. The mixture was evaporated to dryness in vacuo, the brownish solid was suspended in water, and the mixture was basified to pH = 9.5−10 with 0.5% NaOH. Basic byproducts were extracted with Et_2O, and the aqueous layer was acidified to pH 2 by 0.6 N HCl. Upon standing for 12 h, the mixture deposited a white solid which was removed by filtration, washed with a small amount of cold water, and vacuum-dried for 12 h to give 10.6 mg (75%) of pure **3a**, (9*R*)-methylbiotin: $[\alpha]^{23}_D$ +80 (*c* 0.013, pH = 9 NH_4OAc buffer), $[\alpha]^{23}_D$ +14 (*c* 0.20, MeOH); mp 201−205 °C; IR (KBr) 3362, 2923, 1680 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 4.49 (dd, J = 7.7, 4.7 Hz, 1 H), 4.30 (dd, J = 7.9, 4.5 Hz, 1 H), 3.21 (m, 1 H), 2.93 (dd, J = 12.8, 5.0 Hz, 1 H), 2.70 (d, J = 12.7 Hz, 1 H), 2.42 (m, 1 H), 1.80−1.40 (m, 6 H), 1.15 (t, J = 7.0 Hz, 3 H); ^{13}C NMR (125 MHz, CD_3OD) δ 180.61, 166.15, 63.43, 61.62, 57.06, 41.04, 40.53, 34.82, 29.78, 28.07, 17.65; HRMS calcd for $C_{11}H_{18}N_2O_3S$ 258.1038, found 258.1034.

(32) Kuhn, B.; Kollman, P. A. *J. Med. Chem.* **2000**, *43*, 3786−3791.

(9*S*)-Methylbiotin (3b). By the above procedure used for the 9*R* isomer, (9*S*)-methylbiotin (*N*-(*S*-α-phenethyl))amide, **9b** (20.0 mg, 55 μmol) afforded 10.1 mg (67%) of pure **3b**, (9*S*)-methylbiotin: $[\alpha]^{23}_D$ +127 (*c* 0.010, pH = 9 NH_4OAc buffer), $[\alpha]^{23}_D$ +76 (*c* 0.11, MeOH); mp 177−180 °C; IR (KBr) 3291, 2913, 1705, 1688, 1676 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.51 (dd, *J* = 7.6, 4.9 Hz, 1 H), 4.31 (dd, *J* = 7.8, 4.4 Hz, 1 H), 3.20 (m, 1 H), 2.93 (dd, *J* = 12.8, 4.9 Hz, 1 H), 2.70 (d, *J* = 12.7 Hz, 1 H), 2.42 (m, 1 H), 1.80−1.40 (m, 6 H), 1.15 (t, *J* = 6.9 Hz, 3 H); ^{13}C NMR (125 MHz, CD_3OD) δ 180.68, 166.12, 63.34, 61.59, 56.93, 41.01, 40.49, 34.70, 29.58, 27.93, 17.57; HRMS calcd for $C_{11}H_{18}N_2O_3S$ 258.1038, found 258.1033.

Properties for Additional [9*R* and 9*S*]-Alkylated Biotin Methyl Esters. The method used for α-methylation of biotin methyl ester was applied to prepare the following products, which were not further investigated.

[9*R* and 9*S*]-Ethylbiotin Methyl Ester (8b). Prepared from biotin methyl ester by use of KHDMS and ethyl iodide. Yield 49%; $[\alpha]^{23}_D$ +53 (*c* 0.15, $CHCl_3$); R_f 0.24 (5% MeOH−CH_2Cl_2); mp 141 °C; IR (KBr) 3235, 2921, 1728, 1698, 1455 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.59−5.39 (m, 2 H), 4.51 (m, 1 H), 4.31 (m, 1 H), 3.69 (s, 3 H), 3.16 (m, 1 H), 2.92 (dd, *J* = 12.7, 4.8 Hz, 1 H), 2.74 (d, *J* = 12.8 Hz, 1 H), 2.33 (m, 1 H), 1.80−1.40 (m, 8 H), 0.89 (t, *J* = 7.3 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$ δ 176.60, 176.56, 163.40, 61.97, 61.73, 60.08, 55.42, 55.16, 51.45, 46.99, 46.77, 40.52, 31.88, 31.66, 28.64, 28.22, 27.03, 26.56, 25.49, 25.38, 11.79; HRMS calcd for $C_{13}H_{22}N_2O_3S$ 286.1358, found 286.1354.

[9*R* and 9*S*]-Butylbiotin Methyl Ester (8c). Prepared from biotin methyl ester by use of KHDMS and butyl iodide. Yield 19%; $[\alpha]^{23}_D$ +45 (*c* 0.14, $CHCl_3$); R_f 0.24 (5% MeOH−CH_2Cl_2); mp 122−123 °C; IR (KBr) 3370, 2917, 1728, 1696, 1455 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.48−5.31 (m, 2 H), 4.51 (m, 1 H), 4.31 (m, 1 H), 3.69 (s, 3 H), 3.14 (m, 1 H), 2.92 (dd, *J* = 12.7, 4.9 Hz, 1 H), 2.74 (d, *J* = 12.8 Hz, 1 H), 2.35 (m, 1 H), 1.90−1.20 (m, 12 H), 0.89 (t, *J* = 6.9 Hz, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.83, 176.72, 163.31, 61.96, 61.69, 60.08, 55.39, 55.13, 51.46, 45.45, 45.21, 40.53, 32.30, 32.10, 29.57, 28.65, 28.21, 27.05, 22.58, 13.93; HRMS calcd for $C_{15}H_{26}N_2O_3S$ 314.1664, found 314.1679.

[9*R* and 9*S*]-Benzylbiotin Methyl Ester (8d). Prepared from biotin methyl ester by use of KHDMS and benzyl bromide. Yield 23%; $[\alpha]^{23}_D$ +50 (*c* 0.17, $CHCl_3$); R_f 0.28 (5% MeOH−CH_2Cl_2); mp 101−102 °C; IR (KBr) 3409, 2899, 1719, 1711, 1694, 1663, 1445 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.31−7.14 (m, 5 H), 5.41 (br m, 1 H), 5.19 (br m, 1 H), 4.49 (m, 1 H), 4.29 (m, 1 H), 3.62 (s, 3 H), 3.13−2.69 (m, 6 H), 1.90−1.40 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.91, 175.82, 163.21, 139.12, 128.83, 128.41, 126.38, 61.97, 61.63, 60.06, 55.30, 55.09, 51.58, 47.32, 47.12, 40.59, 40.49, 38.53, 38.42, 31.74, 31.59, 28.52, 28.13, 26.88, 26.47; HRMS calcd for $C_{18}H_{24}N_2O_3S$ 348.1508, found 348.1504.

[9*R* and 9*S*]-Allylbiotin Methyl Ester (8e). Prepared from biotin methyl ester by use of KHDMS and allyl bromide. Yield 35%; $[\alpha]^{23}_D$ +55 (*c* 0.19, $CHCl_3$); R_f 0.24 (5% MeOH−CH_2Cl_2); mp 122−123 °C; IR (KBr) 3403, 2905, 1725, 1692, 1676, 1449 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 5.72 (m, 1 H), 5.55 and 5.48 (2 s, 1 H), 5.31 (m, 1 H), 5.04 (m, 2 H), 4.51 (m, 1 H), 4.31 (m, 1 H), 3.68 (s, 3 H), 3.14 (m, 1 H), 2.95 (dd, *J* = 12.8, 4.9 Hz, 1 H), 2.74 (d, *J* = 12.8 Hz, 1 H), 2.60−2.20 (m, 3 H), 1.80−1.30 (m, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.94, 175.83, 163.31, 135.24, 116.90, 61.96, 61.72, 60.07, 55.36, 55.12, 51.55, 45.05, 44.82, 40.57, 40.51, 36.46, 36.36, 31.58, 31.37, 28.59, 28.19, 26.88, 26.43; HRMS calcd for $C_{14}H_{22}N_2O_3S$ 298.1351, found 298.1347.

C. Determining Relative Equilibrium Dissociation Constants. A recently developed assay to measure the streptavidin binding free energy of a ligand relative to biotin was used to characterize the binding affinities of the methylbiotin derivatives. In a mixture of the competing ligand and tritiated biotin, the amount of unbound biotin in solution at equilibrium can be determined by precipitating the protein bound ligands. A competition curve was generated by varying the concentration of the competing ligand, keeping the tritiated biotin and streptavidin concentrations constant, and determining the amount of unbound biotin. Streptavidin was added to a final concentration of 9 nM to mixtures of ^3H-biotin (Amersham, Chicago, IL) at 10 nM and the competing ligand in phosphate buffered saline (50 mM NaH_2PO_4, 100 mM NaCl, pH 7). The concentration of the competing ligand was varied from 0 nM to 50 × ΔK_d × 10 nM, where ΔK_d is the ratio of the streptavidin equilibrium dissociation constants for competing ligand to biotin. The solution containing the protein and ligands was equilibrated over several half-lives for the wild-type streptavidin−biotin dissociation rate ($t_{1/2}$ is 4.7 h at 37 °C) with typical incubation times of 24 h at 37 °C. After equilibration, 50 μL aliquots were added to 200 μL of 0.2 M $ZnSO_4$ followed by addition of 200 μL of 0.2 M NaOH to precipitate the protein bound ligands. The supernatant was assayed by for radioactivity in a liquid scintillation counter (LS-7000, Beckman, Fullerton, CA).

The amount of unbound ^3H-biotin was plotted as a function of the total concentration of competing ligand, with the streptavidin and total ^3H-biotin concentrations kept constant. The solution to the quadratic expression shown in eq 7 was applied to the data with the ratio of the dissociation constants ($\Delta K_d = (K_C/K_L)$) as a fitting parameter using nonlinear chi square minimization (Igor Pro, Wavemetrics, Inc., Lake Oswego, OR).

$$\left(\frac{K_C}{K_L} - 1\right)[L]^2 + \left(\frac{K_C}{K_L}(P_T - L_T) + C_T - P_T + 2L_T\right)[L] + (P_T - C_T - L_T)L_T = 0 \quad (7)$$

where

$$K_C = \frac{[P][C]}{[PC]} = \text{equilibrium dissociation constant for streptavidin-competing ligand complex}$$

$$K_L = \frac{[P][L]}{[PL]} = \text{equilibrium dissociation constant for streptavidin−biotin complex}$$

[C] = concentration of competing ligand

[L] = concentration of ^3H-biotin

[P] = concentration of wild-type streptavidin

Equation 7 was derived from the ratio of the equilibrium dissociation constants of the ligand and biotin−streptavidin complexes, the mass balances for both ligands, and the protein. The derivation assumes that there is negligible free streptavidin, which is true under these experimental conditions.

Results

A. Theoretical Calculations. The type of information typical of a PROFEC analysis is displayed in Figure 1. In this case, the three atoms of the α-methylene group have been used to anchor the set of grid points. The surface displayed is the 0.0 kcal/mol free energy contour. As can be seen from this image, one of the two methylene hydrogen atoms penetrates the surface. This leads to the rejection of this site as candidate for substitution. The other hydrogen atom, however, is enclosed in a small region of "favorable" interaction free energies. This site may admit substitution by a small functional group. The electrostatic character of the site, indicated by the color of the contour surface, suggests that a neutral functional group would be the best choice. The surface colors can range from red at the negative end of the charge spectrum to blue at the positive end. The colors represent the receptor charge distribution and so should be matched by complementary charge distributions on the added

Table 1. Calculated Binding Free Energy Changes Relative to Biotin (kcal/mol)

final structure	ΔG_{prot} (ΔG_2)	ΔG_{solv} (ΔG_1)	ΔG_{tot} ($\Delta G_2 - \Delta G_1$)
	Streptavidin 100 ps forward		
9*R*-methylbiotin	+0.2	+3.0	−2.8
9*S*-methylbiotin	+2.9	+3.2	−0.3
8*S*-methylbiotin	+0.9	+3.3	−2.4
N_1-methylbiotin	+9.8	−0.4	+10.2
	Streptavidin 200 ps[a]		
9*R*-methylbiotin	+0.05 ± 0.1 (2.2)[b]	+2.95 ± 0.3 (2.3)[b]	−2.9 ± 0.4 (−0.1)[b]
9*S*-methylbiotin	+3.18 ± 0.6	+2.85 ± 0.5	+0.3 ± 1.1
8*S*-methylbiotin	+1.31 ± 0.3	+2.85 ± 0.3	−1.5 ± 0.6
	avidin 100 ps forward		
9*R*-methylbiotin	−0.6	+3.0	−3.6
	Avidin 200 ps[a]		
9*R*-methylbiotin	−1.33 ± 0.3	+2.95 ± 0.3	−4.3 ± 0.6

[a] Average is reported with the forward and backward values described by the ± entry. [b] Calculation repeated after experiment was done using the same conformation (t,t,t,g$^-$) for the side chain of biotin. These were 150 ps simulations run only in the forward direction.

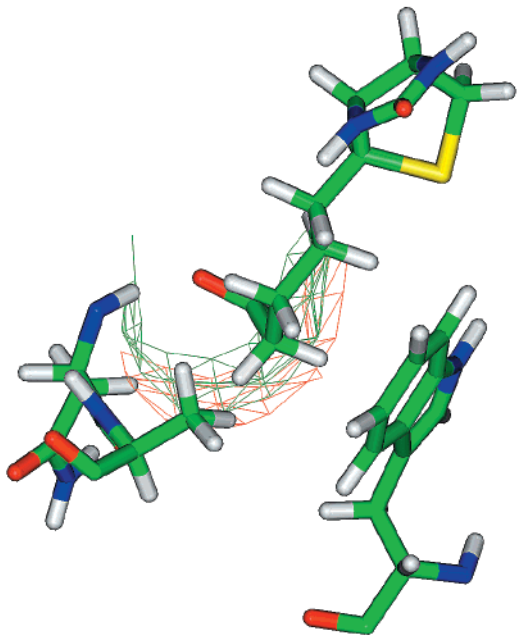


Figure 1. PROFEC contour around biotin. Note that the CO_2^- group is somewhat hidden and that the *pro*-9*R* hydrogen atom is within a favorable (green) contour while the *pro*-9*S* hydrogen atom is protruding into an unfavorable (red) region.

functionality, red matched with positive and blue with negative. The basically green contour surface in the PROFEC image, consistent with the predominantly hydrophobic character of the streptavidin binding pocket, leads to the proposal of a methyl group as the added functionality at this point. This type of analysis was performed for all regions of the biotin molecule. The only other possible site for adding functionality was the *pro*-*S* hydrogen of the β-methylene group. All other possible choices were already bordering the region of "unfavorable" interaction free energies. This was not a surprising result. In many ways, the surprise was in finding any indication of favorable changes at all. It is for this reason that we are also content to base further free energy simulations on what appear at first glance to be fairly small cavities. The raw simulation data used to generate the PROFEC images are not biased toward any particular changes in the ligand. For a "cavity" to be present in the image, it must form naturally during the course of the simulation, without any help from knowledge of the proposed end state. This may lead to smaller cavities than expected given the size of the free energy change observed for the proposed modifications.

Given the results of the PROFEC analysis, the following free energy calculations were undertaken. First, we studied methyl substitution of the *pro*-9*R* hydrogen and of the *pro*-8*S* hydrogen of biotin. The introduction of chiral centers can often increase the complexity of the synthesis of a target compound, so the 9*S* diastereomer of methylbiotin was also included in these studies. A final target compound, *N*-methylbiotin, was chosen as an example of a clearly unfavorable analogue. In addition to the PROFEC analysis, which led to the rejection of this site, this atom is involved in a hydrogen bond that will be lost upon methylation. The increased size of the ligand may also disrupt the important hydrogen bond network to the ureido oxygen. And finally, the X-ray conformation of the ligand has the valeric acid "tail" of the biotin near the area the added methyl group will occupy. There may well be an unfavorable steric effect upon addition of the methyl group in the binding pocket. The calculated binding free energies of these compounds relative to biotin are reported in Table 1. Also reported in this table is the relative binding free energy of the 9*R*-methylbiotin analogue to the protein avidin.

As can be seen from the free energy data of Table 1, the 9*R* methyl analogue of biotin is predicted to bind more strongly to the protein streptavidin than biotin. In addition, 9*R*-methylbiotin is also predicted to bind more tightly to the protein avidin than biotin. This increase in affinity, particularly for the protein streptavidin, is almost completely due to changes in the solvation properties of the ligands. It is therefore unlikely that these improvements could have been predicted simply from visual inspection of the binding pocket.

Are the methyl-substituted biotins significantly less soluble than biotin as suggested by the ΔG_{solv} values in Table 1? Simple continuum approaches to solvation free energy calculations support this claim. Results of calculations with the semiempirical solvation model SM2[33] are reported in Table 2 for the molecules considered in our free energy studies. As can be seen from this table, the continuum methods reproduce the trends observed in our simulations.

B. Synthesis of 9*R*-Methylbiotin 3a and 9*S*-Methylbiotin 3b. The first synthesis of α-methylbiotin (αMB) in racemic form was done by Hanka et al.[21] Treatment

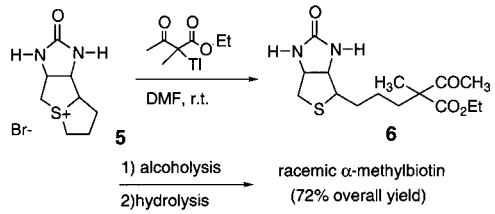
(33) Cramer, C. J.; Truhlar, D. G. *J. Comput.-Aided Mol. Des.* **1992**, *6*, 629−666.

Table 2. Relative Solvation Free Energies for Biotin and Analogues

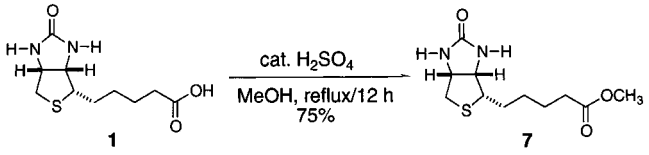
molecule	ΔG_{solv} (SM$_2$)[a]	ΔG_{solv} (TI)[b]
biotin	0.0	0.0
9*R*-methylbiotin	+1.9	+2.95
9*S*-methylbiotin	+1.6	+2.85

[a] Using the SM2 solvation model.[33] [b] Free energy calculations using thermodynamic integration (this study).

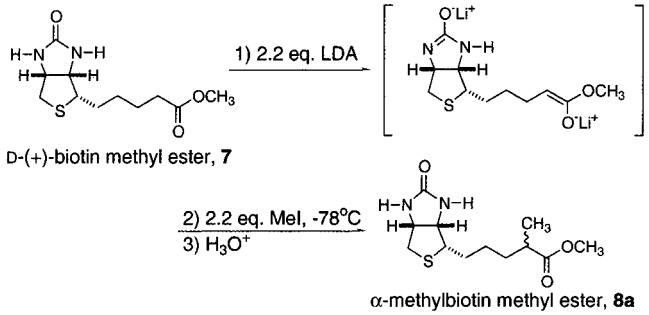
Scheme 1



Scheme 2



Scheme 3



of racemic thionium salt **5** with the thallium salt of ethyl methylacetoacetate at room temperature in DMF afforded **6**, the precursor of α-methylbiotin **3** (Scheme 1). Alcoholysis of the acetyl group followed by hydrolysis of the resulting ester provided racemic methylbiotin in 72% overall yield.

Our synthesis started from readily available D-(+)-biotin **1**. D-(+)-Biotin methyl ester **7** was prepared by Fischer esterification (Scheme 2).[34]

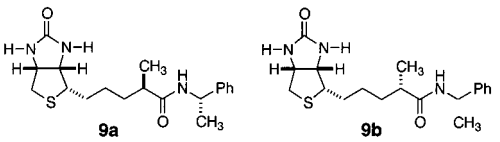
The next step was C-alkylation of D-(+)-biotin methyl ester with MeI. We envisioned that treatment of D-(+)-biotin methyl ester with 2.2 equiv of LDA followed by trapping the resultant dianion with 2.2 equiv of MeI would give diastereomeric mixture of α-methylbiotin methyl ester **8a** in one step (Scheme 3).[35−37]

In our first trial, when D-(+)-biotin methyl ester was treated with 2.2 equiv of LDA in THF at −70 °C and quenched with 10 equiv of CH_3I, only *N*-methylated D-(+)-biotin methyl ester was obtained from the ^{1}H NMR (Table 3, entry A). With the possibility of trianion generation in mind, excess (4 equiv) LDA was used, but α-methylbiotin methyl ester was obtained in a relatively low yield (Table 3, entry B). When TMEDA was employed as an additive with the bulky base LiHMDS, however, α-methylation occurred smoothly at −74 °C after stirring for 2 h (Table 3, entry C). Better results were obtained when the countercations of HMDS bases were varied,[38−40] and KHMDS was found to be the most effective base in α-methylation (Table 3, entries D, E). α-Methylbiotin methyl ester was separated from the starting material by preparative HPLC on C18 column.[41]

The reaction conditions (KHMDS/TMEDA) used in α-methylation were found to be effective with other electrophiles. α-C-alkylation with different alkyl halides and benzaldehyde was tried to make various α-alkylated D-(+)-biotin methyl esters **8b**−**f** (Table 4). Primary alkyl iodides reacted slowly to give α-ethyl D-(+)-biotin methyl ester (**8b**) and α-butyl D-(+)-biotin methyl ester (**8c**) in 49% and 19% respectively (Table 4, entries A, B). For the α-ethylation, longer reaction times (up to 6 h at −74 °C) and use of more HMPA (up to 0.6 mL in 0.1 mmol scale) were tried, but the reaction conversion was not improved. The enolate of D-(+)-biotin methyl ester was found to be relatively unreactive toward C-alkylation. Alkylation with *sec*-alkyl iodide such as isopropyl iodide was also tried; however, the reaction was sluggish and the conversion was about 5%. For the reactive electrophiles such as allylic halides or benzaldehyde, the yield was relatively low because of the formation of N-alkylated side product (Table 4, entries D−F).

α-Methylbiotin methyl ester was hydrolyzed with a mixture of TFA/H_2O (3:1) to give α-methylbiotin **3a**/**3b** in 56−62% yield.[42]

Diastereomers of α-methylbiotin methyl ester or α-methylbiotin were not separable on TLC. Although diastereomers of α-methylbiotin methyl ester were separable on a chiral HPLC column (Cyclobond I 2000 column, Astec Inc.), the separation was not good enough to allow us to perform preparative HPLC. Therefore further derivatization[43,44] was carried out with α-methylbiotin. Because DCC/DMAP coupling[45] did not work well, α-methylbiotin **3a**/**3b** was coupled with 1*S*-(−)-α-methylbenzylamine using BOP-reagent[46] to give (9*R*)-methyl amide **9a** and (9*S*)-methyl amide **9b** in 87 and 60% yield, respectively, after preparative HPLC purification.



Specific rotation values and ^{1}H NMR spectra provided evidence necessary for assigning structures to these

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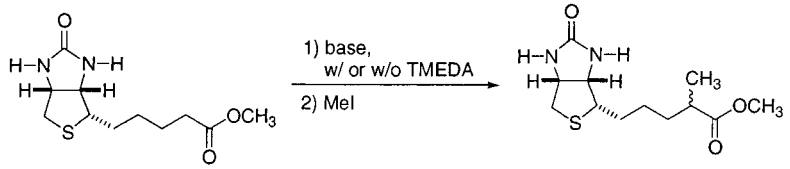
Table 3. Effect of TMEDA and Base on Alkylation of Biotin Methyl Ester

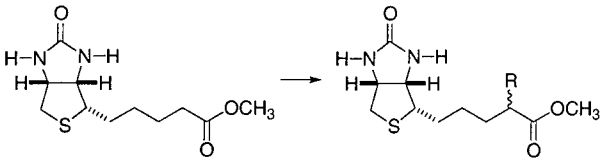


(+)-biotin methyl ester, **7** α-methylbiotin methyl ester, **8a**

entry	base[a]	TMEDA	MeI	% yield/(convn[b])
A	LDA, −78 °C/0.7 h	0 equiv	10 equiv, −60 °C → −50 °C/3.5 h	only *N*-methylation occurred.
B	LDA, −78 °C/0.5 h	0 equiv	1 equiv, −60 °C → −30 °C/5 h	12[c]
C	LiHMDS, −78 °C/1 h	4 equiv	8 equiv, −78 °C → −60 °C/2 h	26/(50:50)
D	NaHMDS, −78 °C/1 h	4 equiv	8 equiv, −78 °C/1 h	59[d]/(80:20)
E	KHMDS, −78 °C/1 h	4 equiv	8 equiv, −78 °C/1.5 h	64[e]/(87/13)

[a] 2 equiv (entry A) and 4 equiv (entries B−E) of base was used. [b] Conversion (convn) is expressed as the ratio of product to starting material, as determined by integration of crude reaction mixture. [c] The product contained some Claisen condensation adduct. [d] Referees to crude yield. [e] Isolated yield at 5 mmol (1.25 g) scale. The ratio of diastereomers was 1:1 by ^{1}H NMR.

Table 4. C-Alkylations of D-(+)-Biotin Methyl Ester 7[a]

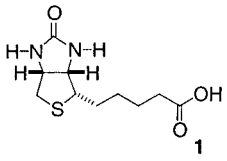
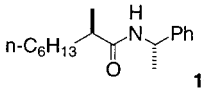
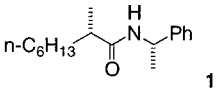
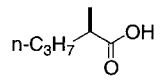
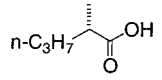


7 **8a-8f**

entry	electrophile	product	% yield/(convn[b])
A	CH_3I	**8**a	64/6:1
B	CH_3CH_2I	**8**b	46/(2:1)
C	*n*-C_4HgI	**8**c	19/(1:2)
D	$C_6H_5CH_2Br$	**8**d	23/(ND[c])
E	CH_2=$CHCH_2Br$	**8**e	35/(ND[c])
F	C_6H_5CHO	**8**f	39/(ND[c])

[a] All reactions were carried out at -78 °C with 4 equiv of KHMDS, 4 equiv of TMEDA and 8 equiv of electrophile. [b] The ratio of product to starting material. [c] Conversion ratio was not determined.

Table 5. Optical Rotations of Some Related Compounds

Structure	Optical Rotation([a]$_D$)	ref.
 **1**	+91.1 (c 1.0, 0.1*N* NaOH)	43
 **10**	-91.6 (c 1.01, $CHCl_3$)	44
 **11**	-63.8 (c 1, $CHCl_3$)[a]	45
 **12**	-18.4 (neat)	46
 **13**	+17.15 (neat)	47

[a] Based on reported optical rotation for the (R,R) compound.

isomeric molecules. We observed that the C-9 methyl group in amide **9b** is more downfield than the C-9 methyl group in **9a**. Helmchen's method[47−49] predicts that the absolute stereochemistry at C-9 should therefore be (*R*) for **9a** and (*S*) for **9b**. Furthermore, the specific rotation of **9a** (−27.3) is more negative than that of **9b** (+12.8). Optical rotation data for model compounds **10-13** (Table 5) show that these *R*-2-methyl acids and amides should have more negative specific rotations the *S*-isomers. The sum of the optical rotations of **1** and **10** is more negative than the sum of the optical rotations of **1** and **11**. We conclude that the absolute stereochemistry at C-9 is (*R*) for **9a** and (*S*) for **9b**.

Each isomeric amide was hydrolyzed with 48% HBr.[50] There was no epimerization at C-9 under these conditions as confirmed by HPLC injection of amide, which was made from the coupling of isolated α-methylbiotin with (*S*)-(−)-α-methylbenzylamine using BOP-reagent. The absolute stereochemistry at C-9 was assigned as (*R*) for the acid **3a** and (*S*) for the acid **3b** from the measurement of optical rotation. The optical rotation values ([α]$_D$) for acid **3a** is +80 and that for acid **3b** is +127. From the argument of the rule of the sum of the optical rotations, the optical rotation of acid **3a,** the 9-(*R*)-methyl isomer, would be more negative than that of acid **3b,** the 9-(*S*)-methyl isomer. In other words, the sum (+73.3) of the optical rotations of compounds **1** and **12** is more negative than the sum (+110.5) of the optical rotations of compounds **1** and **12.** By this reasoning we assigned the absolute stereochemistry at C-9 as (*R*) for acid **3a** and (*S*) for acid **3b.** This has been verified by the X-ray structures for the two molecules complexed to streptavidin.

C. Calorimetric Measurements of Δ*H* of Biotin Binding to Avidin. The calorimetric experiments were performed with the OMEGA titration calorimeter from MicroCal, Inc., Northampton, MA.[51] The titration of avidin with D-(+)-biotin **1**, 9*R*-methylbiotin **3a**, and 9*S*-methylbiotin **3b** was done isothermally at 25 °C. The reference cell was charged with pure distilled water, and avidin solution of known concentration (typically about 1.5 μM) was transferred to the reaction cell. A 250 μL

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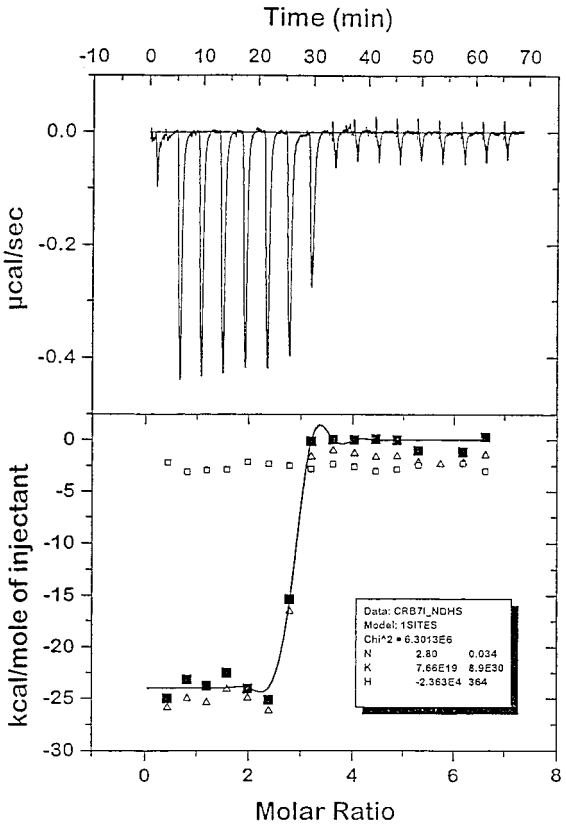

Figure 2. Typical titration curve for calorimetry experiment.

Table 6. Calorimetric Measurements of Δ*H* on Titrating Avidin with Biotin Analogues[a,b]

biotin analogues	Δ*H* (kcal/mol) at pH = 8.91, 25 °C	Δ*H* (kcal/mol) at pH = 7.00, 25 °C
biotin	−23.1 ± 1.1	−23.6 ± 0.4
9*R*-methylbiotin	−17.6 ± 0.4	−14.5 ± 0.2
9*S*-methylbiotin	−16.7 ± 0.4	−14.6 ± 0.2

[a] Values are the average of three measurements. [b] Δ*H* for biotin has been measured to be: (1) −20.3 kcal/mol;[55] (2) −22.5 ± 0.1 kcal/mol.[56]

injection syringe was filled with the solutions (typically 50 μM) of biotin analogues. After thermal equilibrium was reached, the sample was injected as 10−25 μL aliquots at intervals of 3 min. A control experiment with pH = 9 buffer solution or pH = 7 100 mM KCl solution was done to measure the heat of dilution. The heat of binding was determined by best-fit curve. A typical titration diagram is shown in Figure 2, and the results are summarized in Table 6. The Δ*H* of binding of the α-methyl biotins are considerably less exothermic than is biotin.

D. Competitive Binding of Biotin, 9*R*-Methylbiotin, and 9*S*-Methylbiotin to Streptavidin. We used a competition assay to quantitate the equilibrium ΔΔ*G*° of the biotin analogues, relative to biotin at 37 °C. The competitive binding isotherms are shown in Figure 3 and Table 7 summarizes the relative binding affinities and ΔΔ*G*°'s. The 9*R*-methylbiotin displays a decrease in binding free energy of 1.3 kcal/mol relative to biotin, while the 9*S* ligand displays a larger decrease of 2.2 kcal/mol.

Discussion and Conclusion

We have carried out a collaborative theoretical/experimental study, attempting to find a biotin analogue

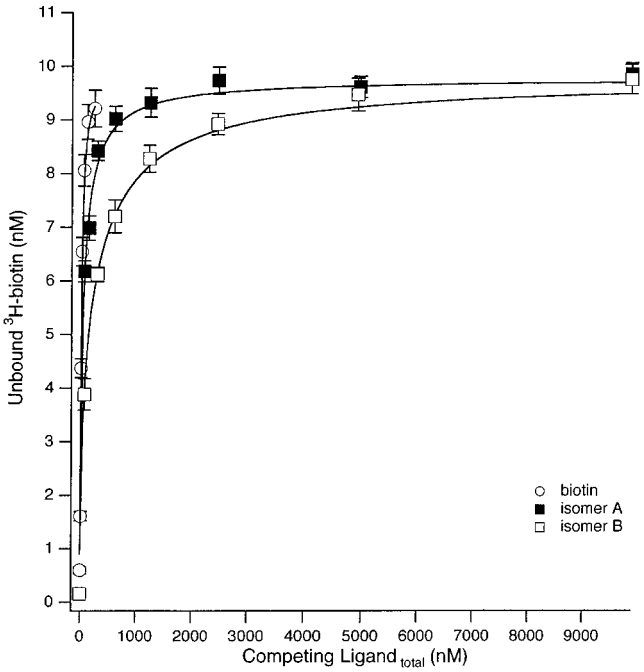

Figure 3. Competition curves at 37 °C for cold biotin and the two 9-methylbiotins competing with ^{3}H-biotin for streptavidin.

Table 7. Streptavidin Binding Affinities of 9-Methylbiotins Relative to Biotin at 37 °C

competing ligand	ΔK_d[a]	ΔΔ*G*° [b] (kcal/mol)
biotin	trial 1:[c] 0.99 ± 0.07	0.0
	trial 2: 1.06 ± 0.07	0.0
methylbiotin isomer A(*R*), **3a**	trial 1: 7.80± 0.60	1.3
	trial 2: 8.00 ± 0.50	1.3
methylbiotin isomer B(*S*), **3b**	trial 1: 34.00 ± 2.00	2.2
	trial 2: 38.00 ± 2.00	2.2

[a] ΔK_d = (K_C/K_L) The standard deviations are estimated from the propagation of errors in multiple sample measurements. [b] ΔΔ*G*° = Δ*G*° (ligand) − Δ*G*° (biotin) = *RT* ln ΔK_d. Characterization was performed at 37 °C. [c] Two independent protein and ligand preparations were tested with multiple samples for each trial.

that binds more tightly to streptavidin/avidin. This study has emphasized the potentially important role of improving the binding of ligands to their targets by reducing their solvation free energy. Such desolvation contributions to binding would not be obvious from simple visualization of macromolecular target structures. We should also note that to our knowledge 9*R*-methylbiotin is the tightest binding analogue of biotin to streptavidin, binding more tightly than the more than 20 analogues previously studied.[5−8]

The calculations predicted that 9*R*-methylbiotin would bind ∼3 kcal/mol more tightly to streptavidin than biotin with this preference coming exclusively from the relative solvation free energies of the ligands. SM2 quantum mechanical calculations also find 9-methylbiotin to be 1.6−1.9 kcal/mol less water soluble than biotin whereas the ΔG_{solv} from the free energy calculations is ∼2.9 kcal/mol. Experimentally, the 9-methylbiotin was indeed found to be significantly less soluble than biotin, although given the limited amounts of material this difference was difficult to quantify. The calculations further predicted that 9*R*-methylbiotin would bind ∼3 kcal/mol more tightly to streptavidin than the 9*S* isomer.While the preference of the 9*R*- over the 9*S*-stereoisomer was

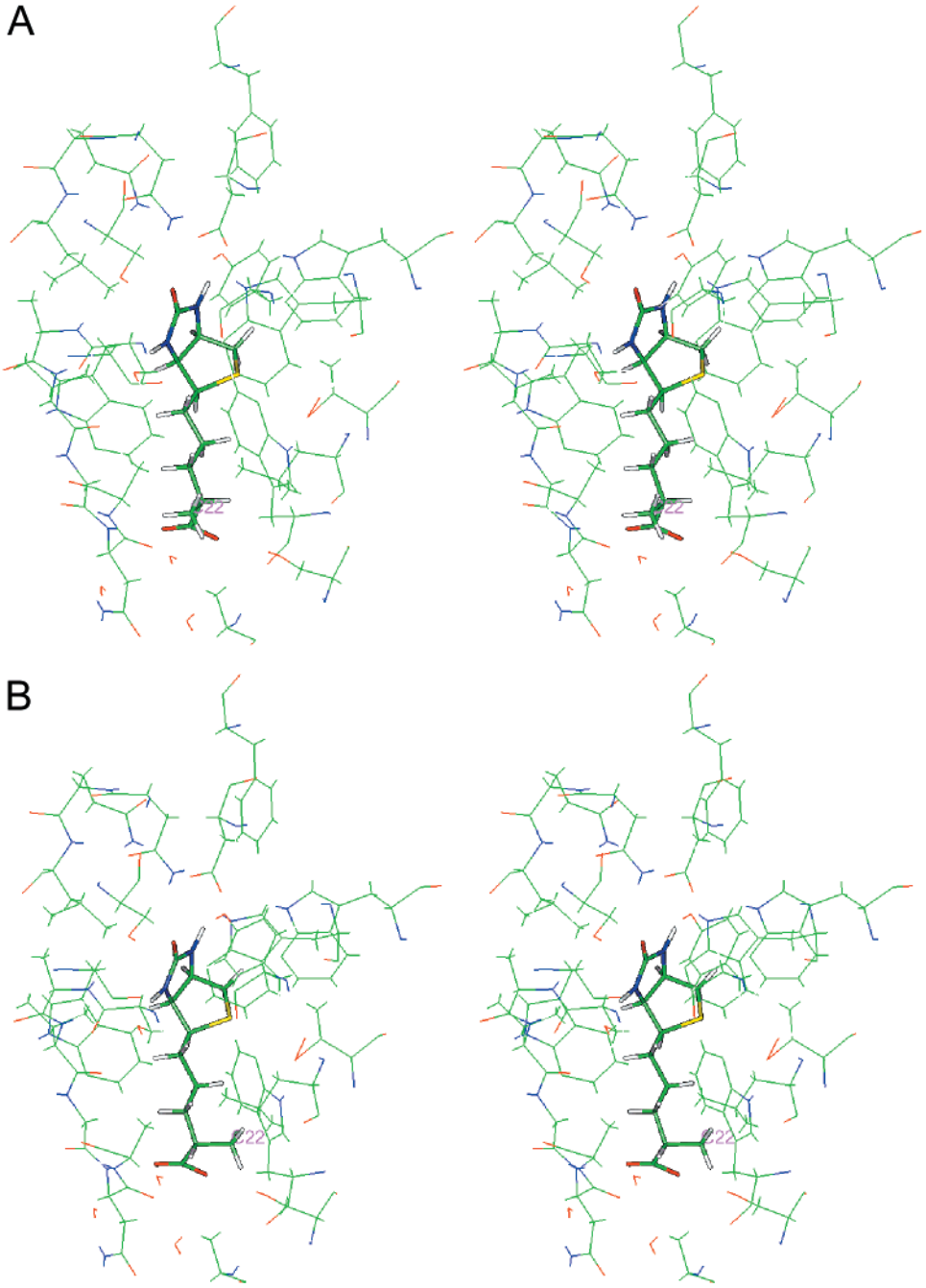

Figure 4. Stereoviews of the complexes of (a) 9*R*- (**3a**) and (b) 9*S*- (**3b**) methylbiotin with streptavidin. The structures are those at the final step of the free energy calculations. C22 corresponds to the α methyl group in both cases.

confirmed by our experiments we find that 9*R*-methylbiotin binds ∼1 kcal/mol less tightly than biotin, not ∼3 kcal/mol more tightly.

Since the experiments showed a discrepancy with the calculated results, we revisited the calculations on the free energy difference between biotin and 9*R*-methylbiotin using different free energy calculation protocols. We first repeated the calculations described in Table 1, running the simulations for 150 ps only in the forward direction and using the same t,t,t,g$^-$ conformation of the biotin side chain as used previously. Instead of 0.05 and 2.95 kcal/mol, we calculated 2.2 and 2.3 kcal/mol, respectively, for ΔG_2 and ΔG_1, which led to a $\Delta\Delta G = -0.1$ kcal/mol, closer to the experimental value of +1.3 kcal/mol. We then used a new approach, MM-PBSA, to estimate the binding free energies of biotin, 9*R*-methylbiotin and 9*S*-methylbiotin to streptavidin. Using 750 ps molecular dynamics trajectories and 100 snapshots (see ref 32 for details), we found free energies of binding of -21.6 ± 0.4, -21.9 ± 0.5, and -19.9 ± 0.4 kcal/mol, respectively, which corresponds to a $\Delta\Delta G$ of −0.3 kcal/mol for 9*R*-methylbiotin relative to biotin. Finally, the X-ray structures of the 9*R*- and the 9*S*-methylbiotin/streptavidin complexes were solved by Behnke and Stenkamp to 1.7 Å resolution.[52] We used these new structures in MM-PBSA calculations to be compared with MM-PBSA calculations using the biotin−streptavidin structure. These calculations led to binding free energies of -20.2 ± 1.1, -21.5 ± 0.8, and -19.7 ± 1.2 kcal/mol for

(52) Behnke, C.; Stenkamp, R., Unpublished crystal structures of 9*R*- and 9*S*-methylbiotin complexed with streptavidin.

biotin, 9*R*- and 9*S*-methylbiotin, and thus to $\Delta\Delta G = -1.3$ kcal/mol for biotin → 9*R* and $\Delta\Delta G = 1.8$ kcal/mol for 9*R* → 9*S*, which is similar to the results obtained with the biotin/streptavidin structure. Thus, one consistently predicts 9*R*-methylbiotin to bind more tightly to streptavidin than biotin, mainly due to its less favorable solvation free energy, although their binding free energy difference varies from −0.1 kcal/mol to −2.9 kcal/mol. On the other hand, the calculated stereoselectivity (*R* vs *S*) ranges from 1.8 to 3.2 kcal/mol compared to the experimental value of 0.9 kcal/mol.

Calorimetry experiments on the two 9-methylbiotins binding to avidin also find a significantly less exothermic ΔH of binding than found for biotin ($\Delta\Delta H = 6-9$ kcal/mol [Table 6]). Assuming a similar $\Delta\Delta G = 1-2$ kcal/mol in avidin and streptavidin, there is clearly significant enthalpy/entropy compensation in the relative binding of biotin and its 9-methyl analogues to (strept)avidin.

At this point, it is not clear why the calculations overestimated the relative binding free energy of 9*R*-methylbiotin and biotin. The conformation around the α−β bond of biotin in streptavidin appears to have the α-methyl group of 9*R*-methylbiotin trans to the γ-CH_2 unit, with the carboxylate in gauche position (Figure 4). This should be a low energy conformer, in contrast to the α-methyl group of 9*S*-methylbiotin where both CO_2^- and α-CH_3 are gauche with respect to the γ-CH_2 unit. These conformations were assumed in the model calculations and supported by the X-ray crystal structures.

The length of the free energy calculations is relatively short, but one might expect with limited sampling that the free energy of growing in a methyl group to be overestimated rather than underestimated. It is clear from the rather large size of ΔG_{solv} that the addition of the α-methyl group is not only a simple "hydrophobic interaction", where Sun et al.[53] found ethane → propane to have a ΔG_{solv} of ∼0.4 kcal/mol, compared to 0.2 kcal/mol experimentally, but perhaps that the α-CH_3 disrupts the solvation of the CO_2^- group. Such α-CH_3 substitutions would also disrupt the solvation of CO_2H groups, given the small ΔpK_a effect observed in comparing primary and secondary carboxylic acids.

Given the fact that the X-ray structure of the 9*R*-methylbiotin/streptavidin complex confirms that the conformations used in the free energy and MM-PBSA calculations are correct, the most plausible explanation for the discrepancy in the calculated $\Delta\Delta G$ is that there is a subtle overestimate of the ΔG_{solv} calculated for adding the 9-methyl group, which is the dominant cause of the negative $\Delta\Delta G$ bind between biotin and 9*R*-methylbiotin. Since the total free energy of solvation of a CO_2^- group is ∼ −70 kcal/mol, it is perhaps not surprising that accurately calculating the ΔG_{solv} for putting a methyl group α to the carboxylate tail is challenging. Future studies are clearly needed to assess this issue. Nonetheless it is possible that a modification of the -$(CH_2)_4$− side chain of biotin that binds more tightly to streptavidin or avidin than biotin may still be found.[54]

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