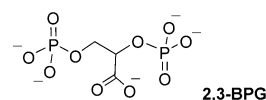


# Controlling the Oxygenation Level of Hemoglobin by Using a Synthetic Receptor for 2,3-Bisphosphoglycerate\*\*

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2,3-Bisphosphoglycerate (2,3-BPG) is an allosteric effector that modulates the oxygenation level of hemoglobin. It is present in human and most other mammalian erythrocytes, normally in an approximately equimolar concentration to that of hemoglobins. It is a glycolytic intermediate resulting from the interconversion between 1,3-phosphoglycerate and 3-phosphoglycerate catalyzed by phosphoglycerate mutase.<sup>[1]</sup> As an allosteric effector, 2,3-BPG regulates the oxygen binding and releasing ability of hemoglobin<sup>[2]</sup> by binding to a central cavity created by the spatial arrangement of the four heme proteins. Specifically, the docking of 2,3-BPG to this cavity decreases the oxygen affinity of hemoglobin, because 2,3-BPG preferentially binds to deoxyhemoglobin.<sup>[3,4]</sup>

Various inherited diseases lead to abnormal concentrations of 2,3-BPG and altered levels of oxygen transport.<sup>[5]</sup> Increased levels of 2,3-BPG are present in patients suffering from hypoxemia and anemia, because this facilitates the oxygen release of oxyhemoglobin into tissue.<sup>[6]</sup> In contrast, high 2,3-BPG levels in individuals with sickle cell anemia are harmful, because 2,3-BPG favors sickling by facilitating the polymerization of sickle hemoglobin.<sup>[7–9]</sup> Controlling the physiological concentration of 2,3-BPG could yield a treatment for these diseases. Our goal was to create a receptor<sup>[10]</sup> that would have a sufficiently high affinity and selectivity for 2,3-BPG to effectively modulate its concentration in physiological media, and thereby control hemoglobin oxygenation.



Herein, we describe a rationally designed synthetic receptor (**1**) that has a highly preorganized cavity composed of a Cu<sup>II</sup> center and four guanidinium groups that serve as binding sites. The receptor displays high affinity and good selectivity for 2,3-BPG. We further show that this receptor will strip the 2,3-BPG from horse hemolyzate, and therefore modulate the oxygenation level of the horse hemoglobin. The

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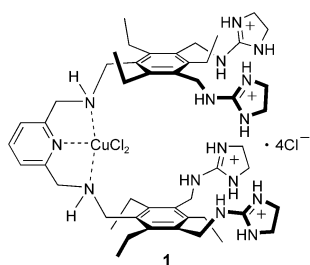
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strategy of sequestering a biological effector with a small synthetic compound is an intriguing approach to controlling physiological function, and represents an alternative to the standard approach of creating small molecules that bind to biological macromolecules, thereby modulating their activity.

2,3-BPG is a highly anionic molecule that is strongly hydrated in water. Hence, several molecular-recognition contacts are required in a receptor to successfully compete with the solvation of this target. Nature competes with solvation by forming several salt bridges between 2,3-BPG and the cationic side chains of lysine and histidine in deoxyhemoglobin.<sup>[4]</sup> Based upon this precedent, receptor **1** was designed. This receptor contains guanidinium groups rather than ammonium and imidazolium groups as with the natural receptor, because molecular recognition entities of guanidiniums are well known to bind phosphates in both biological and synthetic receptors.<sup>[11,12]</sup> Furthermore, receptor **1** has a single Cu<sup>II</sup> binding site, designed to complex the carboxylate or phosphate group of 2,3-BPG. The molecular recognition entities converge creating a clam-shell-shaped cavity. The clam shell is organized upon metal binding. Lastly, the hexa substitution of the two benzene rings in **1** imparts a thermodynamic preference for adjacent groups to be in an up and down pattern,<sup>[13]</sup> thereby imparting convergence of the guanidinium groups prior to binding 2,3-BPG.



Molecular modeling<sup>[14]</sup> shows that **1** has a cavity complementary in size, shape, and charge to 2,3-BPG (Figure 1). The guest slips in between the clam shells, and fits tightly with a large number of binding interactions. The modeling reveals eight hydrogen bonds and five pairs of cation–anion interactions between 2,3-BPG and **1**. Specifically, the carboxylate

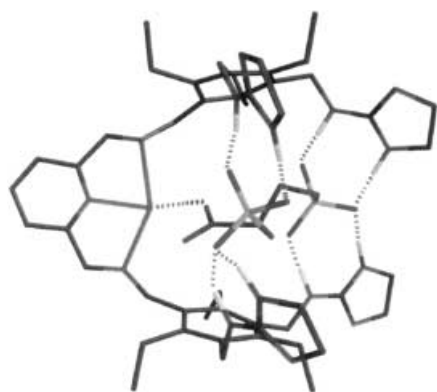
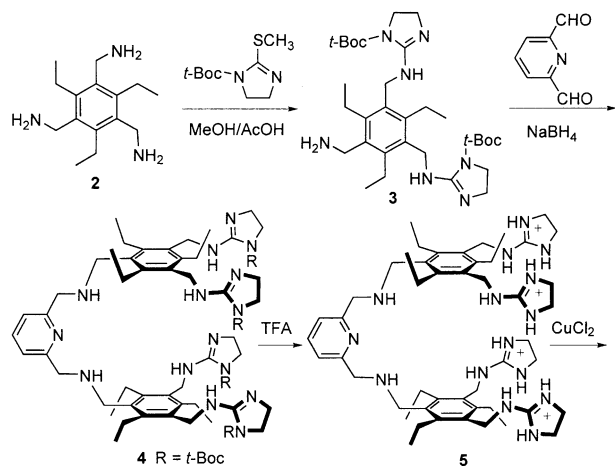


Figure 1. A proposed structure of the complex of **1** and 2,3-BPG.

of 2,3-BPG binds to the Cu<sup>II</sup> center while the phosphates of 2,3-BPG are flanked from above and below by the guanidinium groups.

The synthesis of the receptor is shown in Scheme 1 (see Supporting Information). Triaminomethyltriethylbenzene (**2**)<sup>[15]</sup> was allowed to react with two equivalents of *N*-*t*-Boc-

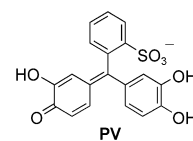


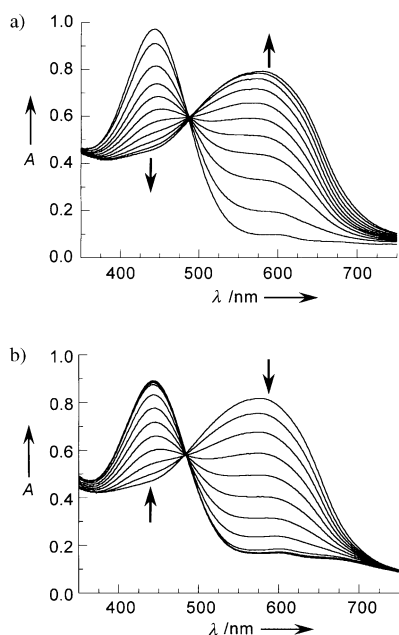
Scheme 1. Synthesis of receptor **1**.

2-methylthio-2-imidazoline<sup>[16]</sup> in MeOH/AcOH, thus giving the bis(*t*-Boc-guanidine)-monoamino-containing structure **3** with a yield of 33 %. Reductive amination of pyridine-2,6-dicarboxaldehyde with **3** by using sodium borohydride gave the *t*-Boc-protected ligand **4** in 76 % yield, which was deprotected in TFA/CH<sub>2</sub>Cl<sub>2</sub> giving the free ligand **5** quantitatively. Finally, the complexation of **5** with one equivalent of CuCl<sub>2</sub> gave the receptor **1**.<sup>[17]</sup>

The molecular recognition behavior of **1** with 2,3-BPG and its analogues was studied with an indicator displacement assay<sup>[18]</sup> by using pyrocatechol violet (PV) in a 1:1 water/methanol solution buffered with 10 mM HEPES at pH 7.4. Figure 2a and 2b show the UV/Vis spectroscopic change of the indicator upon complexation with **1** and displacement from **1** by 2,3-BPG respectively. The binding of PV to **1** causes an absorbance decrease at the  $\lambda_{\text{max}}$  of 445 nm and an increase at a new  $\lambda_{\text{max}}$  of 590 nm with an isosbestic point at 490 nm (Figure 2a), thus yielding a visual change from yellow to blue. The titration data fits well to a typical 1:1 binding algorithm<sup>[19]</sup> with a binding constant of  $1.4 \times 10^6 \text{ M}^{-1}$ . The addition of 2,3-BPG to a solution of the complex between **1** and PV causes a reversal of the spectral response, thus giving an increase at 445 nm and a decrease at 590 nm, and a color change back to yellow. This implies that the indicator was cleanly displaced from the binding cavity of receptor **1** by 2,3-BPG (Figure 2b). This colorimetric response yields a visual response and a quantitative sensing ensemble for 2,3-BPG.

An independent UV/Vis titration of **1** directly with 2,3-BPG in water buffered with 10 mM HEPES at pH 7.4 also shows a 1:1 binding, which changes the Cu<sup>II</sup> d–d absorption. While free **1** has a maximum absorbance at 678 nm with a  $\epsilon =$





**Figure 2.** UV/Vis absorbance spectra of a) 0.054 mM solution of PV in the presence of 0–1.1 equivalents of **1** and b) 0.06 mM **1** and 0.054 mM PV solution in the presence of 0–0.08 mM of 2,3-BPG. Conditions: 1:1 MeOH/H<sub>2</sub>O, 10 mM HEPES buffer, pH 7.4, 25 °C.

210 M<sup>-1</sup> cm<sup>-1</sup>, its 2,3-BPG complex has a maximum absorbance at 660 nm with a  $\epsilon = 170 \text{ M}^{-1} \text{ cm}^{-1}$ . These results indicate that the copper center plays an important role in the binding. Because the binding is very strong, an accurate binding constant was not obtained from this direct titration.

Alternatively, the binding constant of **1** with 2,3-BPG in 1:1 water/methanol (v/v) was calculated to be  $8.0 \times 10^8 \text{ M}^{-1}$  by using the competitive spectrophotometric method<sup>[19]</sup> on the basis of the spectrum changes shown in Figure 2b. Similarly, the binding constants for analogues of 2,3-BPG in the same solvent mixture were measured (Table 1). There is good

**Table 1:** Binding constants with receptor **1** in 1:1 water/methanol at pH 7.4, 25 °C.

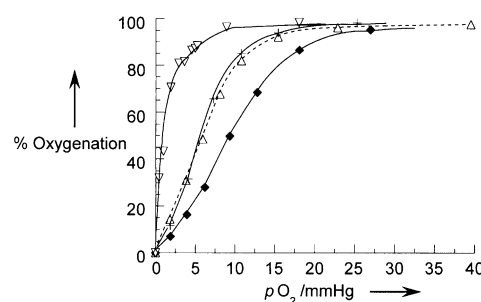
Analyte (as sodium salt)	Binding constant [M <sup>-1</sup> ]
2,3-BPG	$8 \times 10^8$ ( $4 \times 10^{10}$ ) <sup>[a]</sup>
Phospho(enol)pyruvate	$1.3 \times 10^7$
2-Phosphoglycerate	$1.1 \times 10^7$
3-Phosphoglycerate	$4.7 \times 10^6$
$\beta$ -Glycerophosphate	$6 \times 10^4$
Acetate	$7 \times 10^3$

[a] in water at pH 6.8, 25 °C.

selectivity for binding with **1**. For example, the binding constants for the analogues of 2,3-BPG that have one less phosphate are 80 to 180 times lower than that of 2,3-BPG, while the binding constants for simple phosphates and carboxylates are four to five orders of magnitude lower. Apparently, the selectivity is related to the numbers of ion pairs. A binding constant relevant to the use of **1** in blood was desirable for the ultimate goal of this study. However,

displacement of the indicator PV from **1** does not show a perfectly clean isosbestic point in pure water at pH 7.4, possibly because of a small amount of aggregation. To avoid this problem, a slight adjustment of the pH was necessary. At pH 6.8,<sup>[20]</sup> a clean isosbestic point was found, and the binding constant between **1** and 2,3-BPG in water was determined to be  $4 \times 10^7 \text{ M}^{-1}$ . A drop in the binding constant of only 20-fold occurs upon going to pure water from 50 % methanol. Yet, at pH 6.8 the phosphates of 2,3-BPG are protonated to a greater extent than at pH 7.4. Although this binding constant is quite high, it is actually slightly lower than it would be at pH 7.4.

The influence of receptor **1** on the oxygenation levels of hemoglobin was studied spectrophotometrically with a tonometer by using the method of Benesch.<sup>[21]</sup> Figure 3 shows the



**Figure 3.** Oxygenation curves of hemoglobin in diluted hemolyzate of horse red cells (0.04 mM hemoglobin, 10 mM phosphate buffer, pH 7.2, 25 °C). (+) Original hemolyzate, (▽) hemolyzate with 0.16 mM **1**, (◆) hemolyzate with 0.16 mM additional 2,3-BPG, (△) hemolyzate with 0.16 mM additional 2,3-BPG and 0.16 mM **1**.

oxygenation curves of hemoglobin in horse red-cell hemolyzate diluted in 10 mM phosphate buffer solution at pH 7.2. The curve for native hemolyzate (+) has the classic shape found in introductory biochemistry textbooks.<sup>[22]</sup> We first studied the effect of just **1** on the horse hemolyzate. The addition of **1** to the diluted hemolyzate causes the curve to move to lower oxygen pressure (Figure 3▽), thus indicating an increase of the oxygen affinity of hemoglobin. Therefore, as anticipated, the addition of **1** leads to the stripping of the natural 2,3-BPG from hemoglobin. Next, the addition of 2,3-BPG to horse hemolyzate was studied to show that the hemoglobin was responding to 2,3-BPG as it should. Addition of 2,3-BPG was found to decrease the oxygen affinity (◆), as expected. Lastly, the addition of an equivalent of **1** counteracted the added 2,3-BPG, thus revealing that the additional 2,3-BPG was captured by the receptor (△).

All these results are in accordance with the binding affinity we determined for **1**. Because the binding constant of receptor **1** with 2,3-BPG (near  $10^7 \text{ M}^{-1}$ ) is significantly higher than that of hemoglobin with 2,3-BPG (on the order of  $10^4$  to  $10^5 \text{ M}^{-1}$ ),<sup>[3,23,24]</sup> the receptor depletes the 2,3-BPG from the hemoglobin complex and thereby increases the oxygenation level to that of stripped hemoglobin.

In conclusion, we have created a designed synthetic receptor that is highly effective and selective for binding 2,3-BPG, even in physiological media. The affinity is high enough that the receptor can deprive hemoglobin of available 2,3-

BPG, and thus control the active level of 2,3-BPG and the related oxygenation level of hemoglobin. The use of a small organic receptor to bind and regulate the levels of a biological effector, and thereby control a physiological function, represents a rarely explored means towards the development of a pharmaceutical agent.<sup>[25]</sup> Traditionally, small organic molecules are used to bind large biomolecules, and thereby regulate biological function. We believe that the sequestering of small effectors, agonists, and antagonists, by synthetic receptors could play an alternative or complementary role.

### Experimental Section

**Preparation and analysis of hemolyzate:** Fresh defibrinated horse blood (0.6 mL) was centrifuged at 4000 rpm for 5 minutes to isolate the red cells. The supernatant was removed, and the cells were washed four times with 0.9% sodium chloride (1.5 mL each time) in the centrifuge tube. The isolated and washed red cells were hemolyzed by adding distilled water (ca. 1.2 mL). Toluene (ca. 0.3 mL) was added and mixed with the hemolyzate by shaking vigorously. After centrifugation of the sample at 12000 rpm for 10 minutes, the lower clear red layer was transferred by using a pipette to a new centrifuge tube. The toluene-treatment procedure was repeated twice until the interface between the toluene layer and the hemolyzate layer was clear. The hemolyzate was stored in a refrigerator and used within three days. Figure 3 was generated by using the method of Benesch.<sup>[21]</sup>

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- [17] The stoichiometry of the complex of ligand **5** with CuCl<sub>2</sub> was determined to be 1:1 by the mole-ratio method by monitoring the UV/Vis absorbance at 670 nm.
- [18] S. L. Wiskur, H. Ait-Haddou, J. J. Lavigne, E. V. Anslyn, *Acc. Chem. Res.* **2001**, 34, 963–972.
- [19] K. A. Connors, *Binding Constants, The Measurement of Molecular Complex Stability*, Wiley, New York, **1987**.
- [20] At the adjusted pH, the phenols are essentially completely protonated. E. Chiacchierini, R. Gocchieri, L. Sommer, *Collect. Czech. Chem. Commun.* **1973**, 38, 1478–1496.
- [21] R. Benesch, G. Macduff, R. E. Benesch, *Anal. Biochem.* **1965**, 11, 81–87.
- [22] C. K. Mathews, K. E. Van Holde, *Biochemistry*, 2nd ed., Benjamin-Cummings, **1996**, p. 233.
- [23] M. K. Hobish, D. A. Powers, *Proteins Struct. Funct. Genet.* **1986**, 1, 164–175.
- [24] N. Hamasaki, Z. B. Rose, *J. Biol. Chem.* **1974**, 249, 7896–7901.
- [25] For another example, see: C. C. Huval, S. R. Holmes-Farley, G. M. Whitesides (Geltex Pharmaceuticals, Inc.), WO 0038664, **2000**.

- [1] S. I. Winn, H. C. Watson, R. N. Harkins, L. A. Fothergill, *Philos. Trans. R. Soc. London Ser. B* **1981**, 293, 121–130.
- [2] R. Benesch, R. E. Benesch, *Nature* **1969**, 221, 618–621.
- [3] A. Arnone, *Nature* **1972**, 237, 146–149.
- [4] R. Benesch, R. E. Benesch, C. I. Yu, *Proc. Natl. Acad. Sci. USA* **1968**, 59, 526–532.
- [5] M. Delivoria-Papadopoulos, F. A. Oski, A. J. Gottlieb, *Science* **1969**, 165, 601–602.
- [6] R. Juel, *CRC Crit. Rev. Clin. Lab. Sci.* **1979**, 10, 113–146.
- [7] W. N. Poillon, B. C. Kim, O. Castro, *Blood* **1998**, 91, 1777–1783.
- [8] W. N. Poillon, B. C. Kim, R. J. Labotka, C. U. Hicks, J. A. Kark, *Blood* **1995**, 85, 3289–3296.
- [9] M.-C. Garel, N. Arous, M. C. Calvin, C. T. Craescu, J. Rosa, R. Rosa, *Proc. Natl. Acad. Sci. USA* **1994**, 91, 3593–3597.
- [10] For a previous receptor from our group for 2,3-BPG, see M. D. Best, E. V. Anslyn, *Chem. Eur. J.* **2003**, 9, 51–57.
- [11] S. Tobey, E. V. Anslyn in *Kluwer-Plenum Symposium Series on Anion-Recognition*, in press.
- [12] C. L. Hannon, E. V. Anslyn in *Bioorganic Chemistry Frontiers III* (Ed.: H. Dugas), Springer, Heidelberg, **1993**, pp. 193–256.
- [13] D. J. Iverson, G. Hunter, J. F. Blount, J. R. Damewood, K. Mislow, *J. Am. Chem. Soc.* **1981**, 103, 6073–6083.
- [14] Molecular modeling was performed with MacSpartan Pro (Wavefunction, Inc.). The structure was energy-minimized by Merck Molecular Force Field in gas phase.
- [15] L. A. Cabell, M. D. Best, J. J. Lavigne, S. E. Schneider, D. M. Perreault, M.-K. Monahan, E. V. Anslyn, *J. Chem. Soc. Perkin Trans. 2* **2001**, 315–323.
- [16] S. R. Mundla, L. J. Wilson, S. R. Klopfenstein, W. L. Seibel, N. N. Nikolaides, *Tetrahedron Lett.* **2000**, 41, 6563–6566.