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Malaria

Functional Profiling, Identification, and Inhibition of Plasmepsins in Intraerythrocytic Malaria Parasites**

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Malaria is a global disease which affects 300–500 million people annually and kills 1–2 million. The most deadly form of the disease is caused by the pathogen *Plasmodium falciparum*. Currently, quinolines and antifolates are the most common antimalarial drugs.[1] The cost of the drugs, as well as the emergence of multidrug resistance, has, however, become a major problem. Thus, there is a need for newer and ideally cheaper drugs against this devastating disease.[2]

P. falciparum has two stages of growth, one sexual and one asexual. The human asexual erythrocytic phase (blood stage) is the cause of most malaria-associated pathology. Upon their invasion of red blood cells (RBCs), the parasites differentiate (ring stage), metabolize hemoglobin (trophozoite stage), and replicate (schizont stage) over the following 48 h, before being released (by rupture of the host cell) into the blood stream. Proteases, including cysteine (i.e. falcipains) and aspartic proteases (i.e. plasmepsins (PMs)), are required for parasite growth through the digestion of human hemoglobin and the delivery of necessary nutrients. They have long been considered promising antimalarial targets.[3] Genomic data obtained for *P. falciparum* predict at least ten genes that encode aspartic proteases, four of which (PM-I, PM-II, PM-IV, and the histoaspartic protease or HAP) have been found so far, mostly in the food vacuole (FV) of the parasite. The existence of the other hypothetical aspartic proteases, however, has not been confirmed experimentally.

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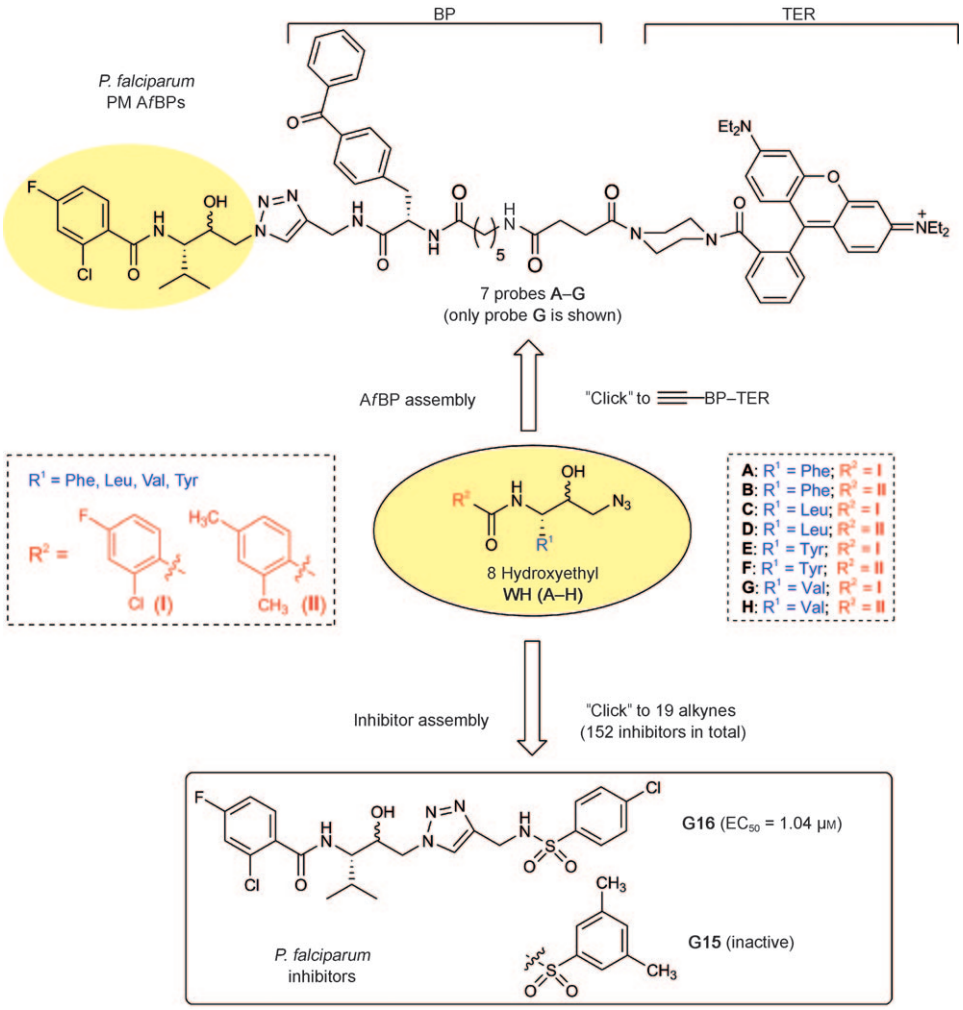
In the last few years, drug-discovery efforts towards potential plasmepsin inhibitors have somewhat waned after gene-knockout experiments showed that parasites could still survive, albeit with a reduced growth rate, without most of the four functionally redundant FV plasmepsins.[4] It is now believed that the only effective way to kill the parasite with PM drugs would be with inhibitors that could simultaneously target as many plasmepsins as possible.[5] At present, most inhibitors developed are only effective against selected PMs[6] owing to difficulties associated with recombinant expression and the insufficient biochemical characterization of certain PMs (i.e. PM-I and HAP) in vitro,[7a,b] as well as the lack of methods that enable the simultaneous screening of the activity of different PMs in situ.[7c] Previously, activity-based probes (ABPs) were used successfully for the in situ screening of malarial cysteine proteases.[8] We report herein the first chemical proteomics approach for the functional profiling of all four PMs in intraerythrocytic malaria parasites. This strategy was made possible by the development of affinity-based probes (A*f*BPs) against PMs (Scheme 1).[9] The in situ screening of PMs with these probes against a focused library of 152 hydroxyethyl-containing small molecules has led to the identification of **G16** as an effective inhibitor against the parasite in infected RBC cultures.

In contrast to other previously known aspartic protease probes, which were based on specific inhibitors against their respective targets (e.g. presenilin and γ-secretase),[10] we aimed to establish a general approach that would be applicable to a variety of aspartic proteases. The seven probes **A**–**G**, each of which contains a hydroxyethyl-based warhead "**WH**" with varying R^1 and R^2 groups, were assembled by click chemistry from the corresponding azide-containing **WH** and the alkyne, which contains a benzophenone (BP) photo-cross-linking unit and a tetraethylrhodamine (TER) reporter (Scheme 1, top).[11] Hydroxyethyl-containing scaffolds are general transition-state analogues of aspartic proteases. In probes **A**–**G**, aliphatic and aromatic amino acid groups were chosen strategically, since they are preferred in three of the four PMs (PM-I, PM-II, and PM-IV; HAP is not well-characterized).[3,4,6] Other aspartic proteases may be targeted in future by structural tuning of the **WH**. The use of click chemistry for the efficient chemical assembly of complex ABPs is well-documented.[12a] In our case, it also provided rapid access to the 152 hydroxyethyl inhibitors **A1**–**H19** against the PMs (see the Supporting Information for complete structures).[12b]

The eight hydroxyethyl **WH**s were synthesized chemically. Upon "click" assembly of the probes and the inhibitors, the resulting compounds were further characterized and purified



Scheme 1. Assembly of affinity-based probes (A*f*BPs) and the 152-membered library of potential inhibitors against all four FV plasmepsins in *P. falciparum*.

(when necessary; see the Supporting Information). To demonstrate the suitability of the probes for the UV-initiated proteomic profiling of plasmepsins, we initially used recombinant PM-I, PM-II, and HAP (Figure 1a; see also Figure S4 in the Supporting Information); highly distinct labeling profiles against different aspartic proteases were observed. This result indicated that the variable R^1 and R^2 groups exerted a strong influence over specific enzyme/probe interactions. The labeling was abolished by treatment with pepstatin and mutations of key catalytic residues in the enzyme active site (see Figures S5 and S6 in the Supporting Information); thus, labeling was indeed dependent on enzyme activity.

The probes were subsequently used to label proteomes of highly synchronized parasites obtained at different stages of parasite development (ring, trophozoite, and schizont; Figure 1b). A 37 kDa protein band, which corresponds to the molecular weight of the four known PMs, was highly visible across probes **A**–**G**, although the labeling intensities varied. Probe **G** consistently gave the strongest labeling profile and thus was chosen for further studies. Analysis by two-dimensional gel electrophoresis/MS and western blotting confirmed unambiguously that the 37 kDa band corresponds to all four labeled PMs (magnification in Figure 1b; see also Figure S8 and Tables S1 and S2 in the Supporting Information). The probes could therefore be used to profile all four known PMs and their enzymatic activities directly from the parasite. The detection of neither zymogens of the four PMs nor other proteins indicated the specificity of the probes in targeting only active PMs. Since it would be highly desirable if the probes could also detect the activity of other previously unidentified aspartic proteases in the malaria proteome (i.e. those predicted by genomic data), a deliberate effort was made in the 2D-GE/MS experiments to look for new protein spots. However, these attempts were unsuccessful; either these hypothetical aspartic proteases were present in very low abundance or our probes were not suitably designed to detect/label them.

Subsequently, we determined the enzymatic activity of the four plasmepsins by labeling both detergent-soluble and detergent-insoluble lysates from each development stage (Figure 1c). The results indicated that PM activity was highly regulated and peaked at the trophozoite stage for the insoluble fraction. This observation is consistent with the role of these PMs in hemoglobin degradation, which occurs in the food vacuole. The distinct profile of soluble-fraction activities peaked at the schizont stage; this peak probably indicates a change in the subcellular localization of the PMs (PM-II was previously shown to be released from the FV in the late schizont stage). Western blotting with antibodies against the four PMs also indicated that their absolute protein expression levels corroborated well with labeling profiles observed with probe **G** (see Figure S9 in the Supporting Information). Thus, the utility of our probes for the accurate reporting of the activities of PMs from crude malaria proteomes was further confirmed.

One of the key advantages of these A*f*BPs is the ability to use them for the simultaneous detection of the activities of multiple PMs in their native environment. This so-called in situ screening method, originally described by Cravatt and coworkers for other enzymatic systems,[13] enabled us to identify potential inhibitors against all four PMs directly from the

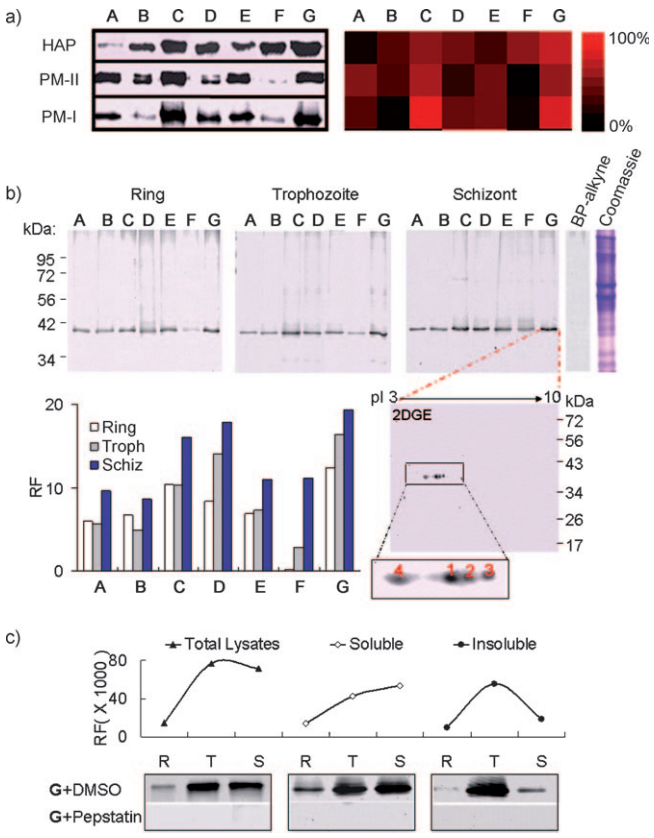


Figure 1. Profiling, identification, and proteome characterization of *P. falciparum* aspartic proteases. a) Characterization of recombinant PMs with the seven *Af*BPs **A**–**G**. Left: labeling profile of recombinant PMs with the *Af*BPs; right: tree-view representation of labeled bands (on the basis of their intensity). b) Parasite extracts at different stages were labeled with the seven *Af*BPs (the TER–BP–alkyne was used as a negative control). Top: in-gel fluorescence scanning showing the specific labeling of the 37 kDa band across different stages; bottom left: spectral counts of the labeled bands; bottom right: schizont-stage parasite extracts were labeled with probe **G** and then subjected to 2D-GE/MS analysis to identify the four PMs (spots 1–4 were identified as PM-II, PM-I, HAP, and PM-IV, respectively). pI = isoelectric point. c) Characterization of plasmepsin activities from total lysates (left), NP-40-soluble fractions (middle), and insoluble fractions (right) across different intraerythrocytic stages of *P. falciparum*. R = ring stage, T = trophozoite stage, S = schizont stage. Top: spectral counts of the respective bands observed by in-gel fluorescence scanning.

malaria proteome without the recombinant production of every active PM.

To identify the most potent inhibitors against all four PMs, we preincubated each compound of the 152-membered hydroxyethyl-based library with the parasite lysate (see Figure S10 in the Supporting Information) and then added probe **G** and subjected the samples to UV irradiation. We determined the relative potency of each inhibitor by measuring the decrease in fluorescence intensity in the 37 kDa labeled band. A total of eight candidate "hits" were identified. (Four of these candidates were identified by screening with individual recombinant PMs by using standard enzymatic assays; the results are not shown.) These eight compounds were resynthesized and purified, and their potency was confirmed in a dose-dependent in situ screening assay (Figure 2a; see also Figure S10 in the Supporting Information). One of the most potent inhibitors identified,

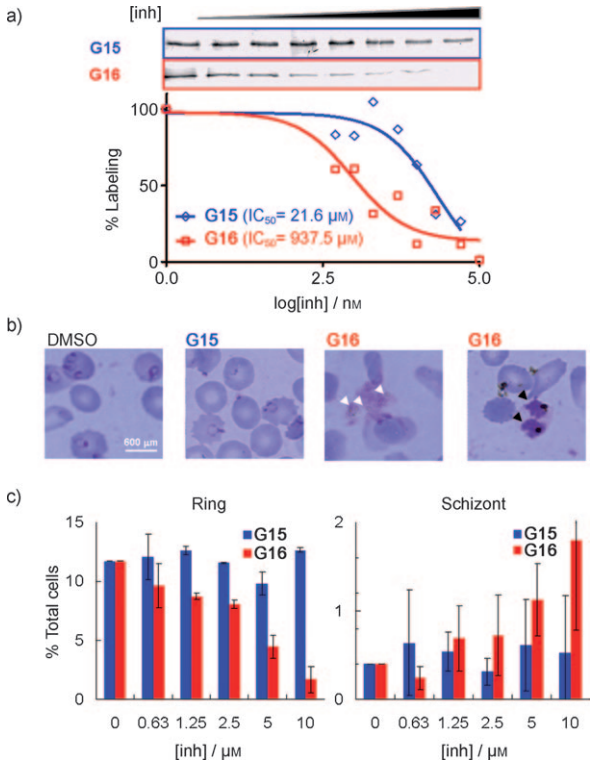


Figure 2. Inhibition of *P. falciparum* aspartic proteases. a) In situ screening assay and determination of the IC_{50} values of **G15** and **G16** against all four PMs in the parasite proteome (for full details, see Figure S10 in the Supporting Information). inh = inhibitor. b) Representative images of parasite-infected RBCs treated with **G16** (10 μM; with dimethyl sulfoxide (DMSO) and **G15** as controls); arrows show the abnormal development of parasites. Scale bar: 600 μM. c) Dose-dependent-inhibition results from (b).

G16, showed an IC_{50} value of 937.5 nM. In contrast, **G15**, a "false positive" identified from standard enzymatic assays by using selected recombinant PMs, showed significantly weaker inhibition (IC_{50} = 21.6 μM). This result underscores the importance of our in situ screening assay for the future discovery of general PM inhibitors.

The inhibitory effect of these candidate compounds was tested with parasite-infected RBC cultures. The distinct activity/solubility profiles of PMs (Figure 1c) prompted us to test the inhibitory effect of the inhibitors against schizont-stage PMs, which showed the highest activity in the detergent nonyl phenoxylpolyethoxylethanol (NP-40). RBCs were treated with the inhibitors 40 h after parasite invasion (i.e. in the late schizont stage). Upon cell rupture, we measured the percentage of parasites in the ring and schizont stages (Figure 2b,c; see also Figure S11 and Table S3 in the Supporting Information). Compound **G16**, but not **G15**, caused a marked decrease in the number of newly formed ring-stage parasites, and at the same time an increase in free extracellular merozoites. Thus, as well as blocking parasite develop-

ment at the trophozoite/schizont stage, as one might expect, the inhibition of PM activity also caused the blockage of either the escape of the parasites from RBCs, or their reinvasion of RBCs. This speculation is further supported by previous findings that PM-II was able to digest an RBC membrane-skeleton protein in the late schizont stage at neutral pH, and that the invasion of *P. falciparum* merozoites was affected by treatment with pepstatin (a general aspartic protease inhibitor).[14] The inhibition of parasites by **G16** was dose-dependent, whereby the estimated EC_{50} value of 1.04 μM was similar to that obtained from the in situ proteomic screening ($IC_{50} = 937.5$ nM). In contrast, **G15** showed little or no inhibition towards infected RBCs, even at the highest concentration tested. Finally, **G16** showed no apparent cytotoxicity against common mammalian cell lines (see Figure S12 in the Supporting Information).

Lastly, to gain insight into the binding mode of **G16** with FV PMs, we carried out molecular-docking studies with three of the four PMs (Figure 3; the X-ray crystal structure of PM-I

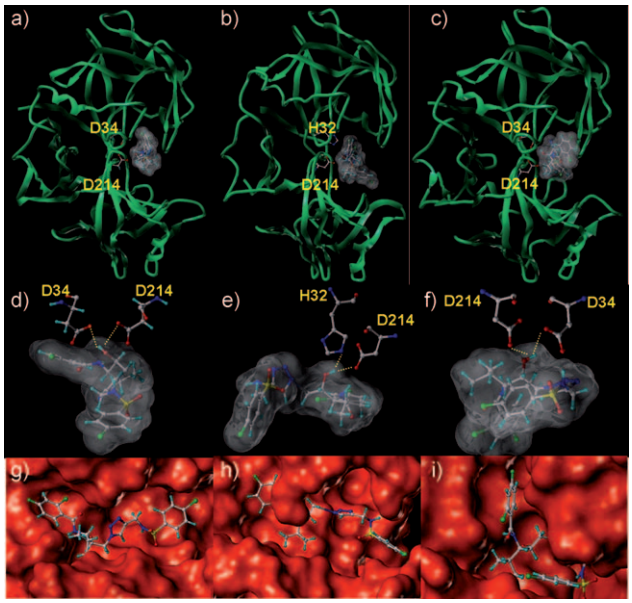


Figure 3. Molecular docking of **G16** in the active site of PM-II, HAP, and PM-IV. **G16** binds to the active-site binding pocket of a) PM-II, b) HAP, and c) PM-IV. The hydroxy group of **G16** interacts closely with d) D34 and D214 in PM-II, e) H32 and D214 in HAP, and f) D34 and D214 in PM-IV. g–i) Preferred conformation of **G16** in the binding pocket of PM-II (g), HAP (h), and PM-IV (i).

has not been reported). The results showed that the molecule binds well in the active site confined by the D34–D214 aspartic acid pair in the structure of PM-II and PM-IV, and by H32 and D214 in the structure of HAP (Figure 3a–c). Detailed analysis of the top 100 docked models identified the preferred conformations of **G16** in PM-II and PM-IV, as well as in nonclassical HAP (Figure 3g–i). The results also showed that the hydroxy group of **G16** interacts closely with D34 and D214 in PM-II and PM-IV, as originally designed (Figure 3d,f). In binding to HAP, **G16** assumed a position in which its hydroxy group fitted between H32 and D214 in the enzyme active site (Figure 3e). A similar interaction was observed previously between the hydroxy group in pepstatin (a general aspartic protease inhibitor) and HAP.[15,16]

In summary, we have developed the first affinity-based probes for the functional profiling of all four PMs in intraerythrocytic malaria parasites. Subsequent in situ screening of parasites with these probes led to the identification of a compound, **G16**, which showed good inhibition against all four PMs and parasite growth in infected RBCs. Molecular modeling indicated that this inhibitor binds to the active site of the plasmepsins tested, as originally designed. Our results indicate the feasibility of using A*f*BP approaches for the identification of inhibitors of less-characterized enzymes (such as HAP) and inhibitors of multiple targets. We anticipate that these new chemical tools should facilitate the discovery of unknown parasite biology and new antimalarial drugs. In the current study, we were unable to detect previously predicted but unidentified aspartic proteases from the malaria proteome. Thus, **G16** might have further targets, other than the four plasmepsins, in the malaria proteome. Future research will focus on the development of new chemical probes to address these issues.

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[1] R. W. Snow, C. A. Guerra, A. M. Noor, S. I. Hay, *Nature* **2005**, *434*, 214–217.
[2] C. Boss, O. Corminboeuf, C. Grisostomi, S. Meyer, A. F. Jones, L. Prade, C. Binkert, W. Fischli, T. Weller, D. Bur, *ChemMedChem* **2006**, *1*, 1341–1345.
[3] a) D. A. Fidock, R. T. Eastman, S. A. Ward, S. R. Meshnick, *Trends Parasitol.* **2008**, *24*, 537–544; b) P. J. Rosenthal, *Int. J. Parasitol.* **2004**, *34*, 1489–1499; c) D. E. Goldberg, *Curr. Top. Microbiol. Immunol.* **2005**, *295*, 275–291.
[4] a) J. Liu, E. S. Istvan, I. Y. Gluzman, J. Gross, D. E. Goldberg, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 8840–8845; b) M. E. Drew, R. Banerjee, E. W. Uffman, S. Gilbertson, P. J. Rosenthal, D. E. Goldberg, *J. Biol. Chem.* **2008**, *283*, 12870–12876.
[5] a) F. Hof, A. Schutz, C. Fah, S. Meyer, D. Bur, J. Liu, D. E. Goldberg, F. Diederich, *Angew. Chem.* **2006**, *118*, 2193–2196; *Angew. Chem. Int. Ed.* **2006**, *45*, 2138–2141; b) A. Nezami, T. Kimura, K. Hidaka, A. Kiso, J. Liu, Y. Kiso, D. E. Goldberg, E. Freire, *Biochemistry* **2003**, *42*, 8459–8464.
[6] K. Ersmark, B. Samuelsson, A. Hallberg, *Med. Res. Rev.* **2006**, *26*, 626–666.
[7] a) H. Xiao, A. F. Sinkovits, B. C. Bryksa, M. Ogawa, R. Y. Yada, *Protein Expression Purif.* **2006**, *49*, 88–94; b) H. Xiao, T. Tanaka, M. Ogawa, R. Y. Yada, *Protein Eng. Des. Sel.* **2007**, *20*, 625–633; c) D. C. Greenbaum, *Trends Pharmacol. Sci.* **2008**, *29*, 51–56.
[8] a) D. C. Greenbaum, A. Baruch, M. Grainger, Z. Bozdech, K. F. Medzihradszky, J. Engel, J. DeRisi, A. A. Holder, M. Bogyo, *Science* **2002**, *298*, 2002–2006; b) S. Arastu-Kapur, E. L. Ponder, U. P. Fonovic, S. Yeoh, F. Yuan, M. Fonovic, M. Grainger, C. I. Phillips, J. C. Powers, M. Bogyo, *Nat. Chem. Biol.* **2008**, *4*, 203–213.
[9] We previously proposed the use of the term A*f*BP for this kind of active-site affinity probe: E. W. S. Chan, S. Chattopadhaya, R. C.

Panicker, X. Huang, S. Q. Yao, *J. Am. Chem. Soc.* **2004**, *126*, 14435 – 14446.
[10] a) A. Weihofen, K. Binns, M. K. Lemberg, K. Ashman, B. Martoglio, *Science* **2002**, *296*, 2215 – 2218; b) Y. Takahashi, Y. Konno, N. Watanabe, H. Miyashita, M. Sasaki, H. Natsugari, T. Kan, T. Fukuyama, T. Tomita, T. Iwatsubo, *ACS Chem. Biol.* **2007**, *2*, 408 – 418.
[11] a) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708 – 2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599; b) C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057 – 3062.
[12] a) M. J. Evans, B. F. Cravatt, *Chem. Rev.* **2006**, *106*, 3279 – 3301; b) K. A. Kalesh, P. Y. Yang, R. Srinivasan, S. Q. Yao, *QSAR Comb. Sci.* **2007**, *26*, 1135 – 1144.
[13] D. Leung, C. Hardouin, D. L. Boger, B. F. Cravatt, *Nat. Biotechnol.* **2003**, *21*, 687 – 691.
[14] a) S. Le Bonniec, C. Deregnaucourt, V. Redeker, R. Banerjeei, P. Grellier, D. E. Goldbergi, J. Schrevel, *J. Biol. Chem.* **1999**, *274*, 14218 – 14223; b) P. Dejkriengkraikhul, P. Wilairat, *Z. Parasitenkd.* **1983**, *69*, 313 – 317.
[15] P. Phaumik, H. Xiao, C. L. Parr, Y. Kiso, A. Gustchina, R. Y. Yada, A. Wlodawer, *J. Mol. Biol.* **2009**, *388*, 520 – 540.
[16] According to the docking results, an *S* configuration was favored for the carbon center attached to the hydroxy group in **G16**; see the Supporting Information for details.

