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Blechnum pyramidatum (Lam.) Urb. is a species of extensive medicinal use in the American continent. In fact, antidiabetic and anticancer preparations from this plant have been patented in Mexico, even though their active constituents are not yet known. It was recently discovered that *B. pyramidatum* inhibits the action of the α -glucosidase enzyme, thereby corroborating the antidiabetic properties attributed to this plant. The primary purpose of this study was to identify and characterize the α -glucosidase inhibitors from this species. Bioassay-guided fractionation of a crude extract of *B. pyramidatum* led to the isolation of a main α -glucosidase inhibitor, Palmitic acid (IC₅₀ 237.5). This compound was identified by both spectroscopic and spectrometric analysis. Its inhibitory activity was similar to that of the antidiabetic drug acarbose (IC₅₀ 241.6 μ M), which was used as a positive control in our bioassay. Kinetic analysis established that palmitic acid acted as a competitive inhibitor. Docking analysis predicted that this compound binds to the same site as acarbose does in the human intestinal α -glucosidase (PDB: 3TOP). The presence of palmitic acid in *B. pyramidatum* and its potent inhibitory activity against α -glucosidase enzyme provides solid evidence to support the antidiabetic use of this plant in traditional medicine.

Keywords: *Blechnum pyramidatum*, α -Glucosidase inhibition, Kinetic analysis, Docking analysis.

Medicinal plants have been systematically used since ancient times, especially in countries where significant ancestral cultures flourished [1]. Despite today's technological advances, medicinal plants continue to be a widely used resource to prevent and treat human diseases. Recently, the World Health Organization estimated that approximately three-quarters of the world's population trust and utilize plants for their health care needs [2]. Faced with this panorama, scientists from all over the world must continue to generate better information to develop recommendations for effective and safe uses of many medicinal plants.

Blechnum pyramidatum (Lam.) Urb. is a species of wide medicinal use in different countries of the American continent. Cooking of the leaves is prescribed to treat skin problems [3]. A decoction of this plant can be administered as a diuretic [4] and as an antiemetic [5]. In some places, this plant is applied to treat snakebites [6]. In Mexico, the antidiabetic and anticancer uses of this plant have been patented, although the active compounds have never been isolated nor identified [7]. Therefore, there is an urgent need to identify the active components of this plant to guarantee its quality and safe use. In a preliminary study, we detected that the organic extract from this plant showed significant inhibitory activity against the α -glucosidase enzyme, although it was not possible for us to fully identify the component(s) responsible for that activity [8]. The latter was partly due to the presence of large amounts of chlorophyll and other related compounds, which interfered with the bioactivity results we obtained in the initial extraction of this plant. Still, obtained results suggests that inhibition of α -glucosidase enzyme is part of antidiabetic mechanisms from this plant.

It is important to point out that conventional bioassays performed to detect α -glucosidase activity typically quantify the enzymatic activity by measuring the absorbance of the reaction mixture at 400 nm [9a-c]. Chlorophyll and related compounds also exhibit intense

absorption peaks at this same wavelength. Therefore, the removal of these types of compounds prior to chemical studies is essential to obtain more accurate results in detecting α -glucosidase bioactivity. For this reason, we proceeded with the removal of chlorophyll and related compounds from the initial crude extract by using activated carbon to avoid interferences in the enzymatic readings.

The aerial parts of *B. pyramidatum* were macerated with a mixture of CHCl₃-MeOH (1:1). Subsequently, this mixture was blended with activated carbon, which was filtered and concentrated to obtain the crude extract. This extract inhibited 69.4 % of the α -glucosidase activity at a final concentration of 6.25 mg/mL. Afterward, the organic extract was dissolved in a hydro-methanol mixture (20:80) and successively partitioned by two solvents, hexane and ethyl acetate, to obtain three major fractions with increasing polarity. Of these three fractions, only the hexane fraction showed promising activity (80.1 % of enzyme inhibition at 6.25 mg/mL). Bioassay-guided fractionation of the hexane fraction allowed the isolation of palmitic acid (compound **1**), as the main active compound from *B. pyramidatum*. This compound was identified by spectroscopic analyses including ESI-HR-MS and NMR (¹H, ¹³C, DEPT 135, DEPT 90, COSY, NOESY, HMBC and HMQC) [8, 10].

Compound **1** inhibited the α -glucosidase enzyme in a concentration-dependent manner with an IC₅₀ value of 237.5 μ M therefore showing similar potency to acarbose (positive control, IC₅₀ 241.6 μ M). During α -glucosidase evaluations, we initially found that palmitic acid only showed moderate activity, which did not correlate with the activity detected in the hexane fraction through which this compound was isolated. This fact allowed us to recognize that palmitic acid had poor solubility in DMSO. There have been previous reports about α -glucosidase inhibition by fatty acids, including palmitic and oleic acids, with DMSO being regularly used to solubilize samples as part of standard protocols to

detect bioactivity [11a-c]. Our evidence suggested that a re-evaluation of the inhibition of the α -glucosidase enzyme by palmitic acid in other solvents was necessary to both determine whether there was an effect by the reduced solubility of palmitic acid in DMSO and to compare our findings to previously reported enzymatic activity. To find a solvent which would allow us to get more reliable results for palmitic acid activity - and, in general, for low polar compounds - we proceeded to evaluate the inhibition of α -glucosidase by some common solvents in different quantities, including ethanol, methanol, propanol, and isopropanol. Through these tests, we detected that ethanol in small volumes only slightly decreased the α -glucosidase activity and that palmitic acid was solubilized better than with DMSO. This finding allowed us to obtain more real inhibition results for low polarity compounds.

We proceeded to evaluate the activity of palmitic acid using ethanol to compare to the results we obtained with DMSO. We also assessed oleic acid since this compound has similar solubility properties to those of palmitic acid, and we also re-evaluated the positive control, acarbose (IC_{50} 217.7 μ M in DMSO). As a result, oleic acid showed potent α -glucosidase inhibitory activity (IC_{50} 38.9 μ M), even better than palmitic acid, and acarbose showed similar potency to that displayed using DMSO (IC_{50} 241.6 μ M in ethanol). This evidence suggests that reported data about α -glucosidase inhibition by certain low polar compounds could be inaccurate due to their poor solubility in DMSO which is used in most standard protocols.

To obtain further evidence of the nature of the interaction of palmitic (1) and oleic acids (2) with α -glucosidase, we carried out kinetic analyses. Lineweaver-Burk plots [12a] were constructed using different concentrations of substrate and fatty acids 1-2 (Figure 1). The results in Figure 2 and Figure 3 indicated that compounds 1-2 showed typical reversible competitive plots, with series of lines having the same y-intercept as the enzyme without inhibitors. These results suggested that compounds 1-2 bind to α -glucosidase or to the substrate-enzyme complex. Acarbose also behaved as a competitive inhibitor [12a]. These results show that both fatty acids are potent competitive inhibitors of the α -glucosidase enzyme.

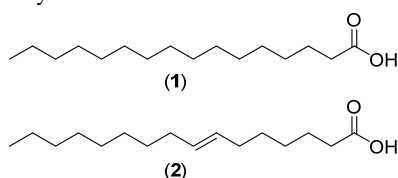


Figure 1: (1) Palmitic acid and (2) Oleic acid.

Taking into consideration the results of the kinetic analysis performed, we conducted a molecular docking study to evaluate the putative binding mode of fatty acids 1-2 into the human intestinal α -glucosidase (PDB: 3TOP). Results indicate that fatty acids bind mainly through hydrophobic interactions. Figure 4 shows the superposition of docking poses of compounds 1-2 and acarbose in the binding site. It is interesting to note that despite the analyzed fatty acids being mainly hydrophobic they bind to the same site as acarbose, which is a more polar compound compared to fatty acids 1-2. As expected, acarbose interacts with the binding site through many hydrogen bonds (Figure 5a) and compounds 1-2 interact mainly via hydrophobic interactions (Figure 5b). However, palmitic acid interacts with Asp 1526 and Arg 1582 through the formation of hydrogen bonds while oleic acid weakly interacts with Lys 1460 by the formation of hydrogen bonds with hydroxyl groups (Figures 6). Finally, in the results of the docking analysis, it is clearly observed that the oleic acid pose fits better than the palmitic acid pose in the

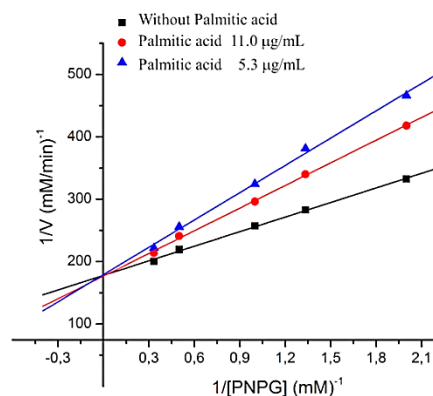


Figure 2: Lineweaver-Burk plot of α -glucosidase inhibition at different concentrations of substrate and palmitic acid.

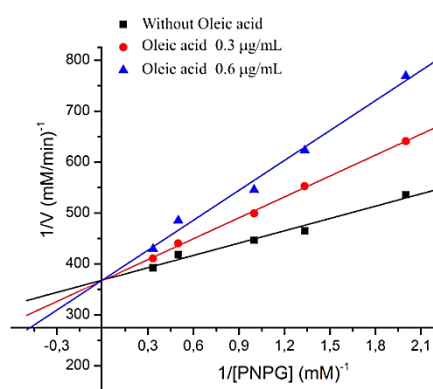


Figure 3: Lineweaver-Burk plot of α -glucosidase inhibition at different concentrations of substrate and oleic acid.

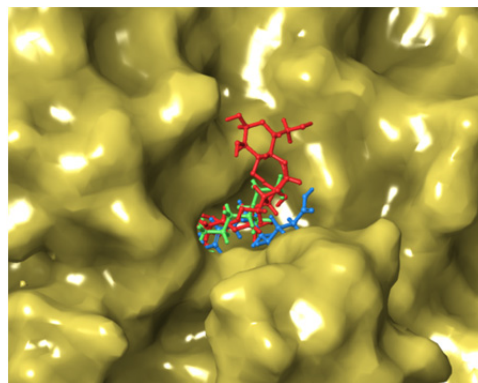


Figure 4: Superposition of docking poses of compounds 1 (in green), 2 (in blue) and acarbose (in red).

α -glucosidase receptor site. This finding suggests that the three-dimensional arrangement due to the presence of the double bond is responsible for the better activity displayed by oleic acid.

In summary, palmitic acid was isolated from the *B. pyramidatum* active fraction against α -glucosidase. Even though this compound was previously reported as a weak inhibitor of α -glucosidase, our data suggests that the use of a polar solvent (DMSO) in the bioassay conditions has produced inaccurate results, especially for low polarity compounds. Oleic acid showed higher activity than both palmitic acid and acarbose. These compounds exhibited a competitive type of inhibition against *S. cerevisiae* α -glucosidase. Therefore, this fatty acid-rich plant might also be an interesting alternative for reducing blood sugar level in people affected by DM.

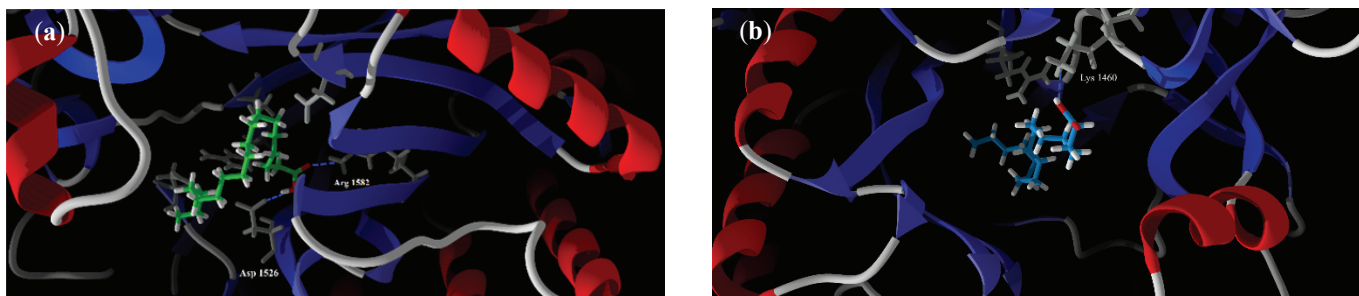


Figure 5: Docking poses of compound 1 (a) and compound 2 (b). H-bond interactions are highlighted.

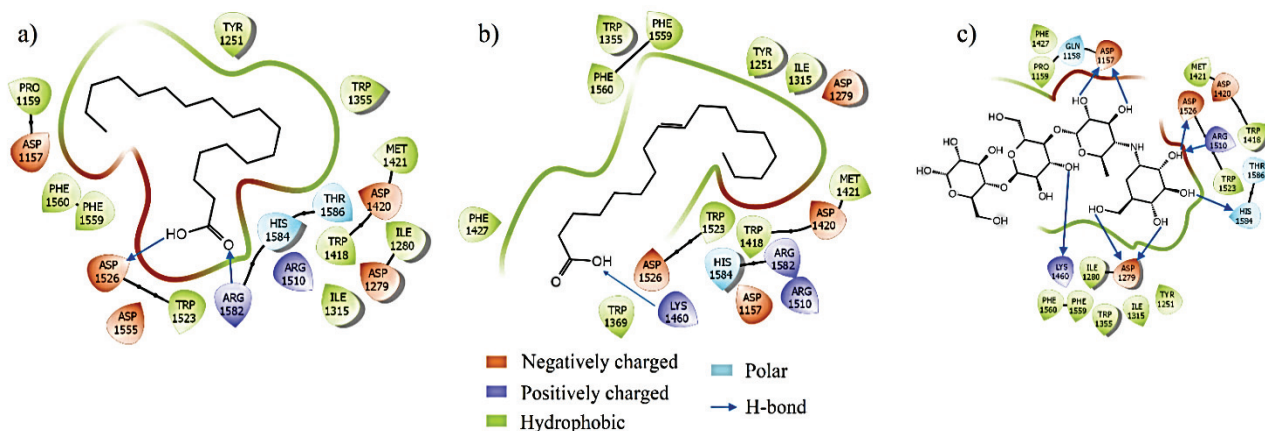


Figure 6: Comparison between the interaction of Palmitic acid (a), Oleic acid (b) and acarbose (c) with α -glucosidase active site.

Experimental

General Experimental Procedures: NMR spectra were acquired on Jeol Eclipse 400 MHz spectrometer (referenced to δ_{H} 7.26, δ_{C} 77.0 for CDCl_3). APCIHR-MS were acquired on a JEOL LC-mate mass spectrometer. The purification of the compounds was carried out on Agilent 1100 HPLC system equipped with a quaternary pump, a diode array detector, and a normal phase silica gel column (Phenomenex Luna, 4.6 mm \times 100 mm, 5 μm) at a flow rate of 1 mL/min. Column chromatography was used with silica gel 60 (70-230 mesh, Merck). TLC (analytical) was performed on pre-coated silica gel 60 F254 plates (Merck). All solvents were HPLC grade and used without further purification.

Plant material and extract preparation: *B. pyramidatum* (Acanthaceae) was collected in Santa Clara, Chiriquí, in the Republic of Panama. This plant was identified by Jorge Lezcano and was deposited to the Herbarium at the University of Panama. The aerial plant parts were dried at room temperature for a week and then were grounded for further study. This material (50.0 g) was extracted by maceration at room temperature with a mixture of CHCl_3 -MeOH (1:1). The solvent mixture was mixed with activated carbon (charcoal; 5 g) and stirred for 20 minutes at room temperature. The solution was filtered through celite in a Büchner funnel. The resulting solution was evaporated under reduced pressure to remove the solvent to leave a semi-solid paste (4.6 g).

Chemical studies: The crude extract was subjected to bioassay-guided fractionation in which the extract was re-suspended in a mixture of water-MeOH 70:30 and sequential partitions were made with Hexanes [Hex] (5 \times 200 mL) and ethyl acetate [AcoEt] (5 \times 200 mL). Each obtained fraction, including the final hydromethanol fraction, was evaporated to dryness and then subjected to enzymatic assay. Hex fraction (active, 1.61 g) was fractionated by column chromatography on silica gel (35 g). The column was eluted with

Hex, followed by a gradient of Hex:EtOAc (1:0 \rightarrow 0:1) and finally with a gradient of EtOAc:MeOH (1:0 \rightarrow 1:1). Altogether, 109 fractions (25 ml each) were collected and combined according to their TLC profiles to yield nine primary fractions (FA to FI), which were re-evaluated against α -glucosidase and the activity was presented in fraction FA [eluted with 100% Hex]. Fraction FA (0.31 g) was further subjected to silica gel column chromatography and eluted with a gradient of Hex:EtOAc (1:0 \rightarrow 0:1). This process led to six fractions (FA-1 to FA-6), which were also evaluated. Fraction FA-2 was eluted with Hex:EtOAc (9:1) to afford 3.1 mg of compound 1, which was the compound responsible for the activity. The other fractions were also evaluated but did not show an inhibitory effect *in vitro* against the enzyme α -glucosidase.

α -Glucosidase inhibitory assay: The α -glucosidase inhibitory assay was performed according to Chan and collaborators [9a], with modifications [12a and b]. α -Glucosidase from baker's yeast was purchased from Sigma Chemical Co. The inhibition was measured spectrophotometrically at pH 7.0 and 37°C employing 2 mM *p*-nitrophenyl α -D-glucopyranoside (PNP-G) as a substrate and 32 mU/mL of the enzyme, in 100 mM potassium phosphate buffer (enzyme stock). Acarbose was dissolved in phosphate buffer, and serial dilutions were prepared (in order to obtain the IC_{50}) and employed as positive control. The absorbance (A) of 4-nitrophenol released by the hydrolysis of PNP-G was measured at 400 nm by Synergy HT Bio Tek microplate spectrophotometer. A 20 μL of acarbose, plant extract or isolated compound solution (in DMSO or Etanol) were incubated for 7 min with 150 μL of enzyme stock at 37°C. After incubating, 150 μL of substrate was added and further incubated for 20 min at 37°C. All assays were performed in 96-well microplates (Greiner bio-one 655101) in triplicate. The activity of samples was calculated as a percentage in comparison to a control according to the following equation:

$$\%Inhibition = \left(\frac{(\Delta A_{control} - \Delta A_{sample})}{\Delta A_{control}} \right) \times 100\%$$

The concentration required to inhibit activity of the enzyme by 50% (IC₅₀) was calculated by regression analysis [13].

Kinetics of α -glucosidase inhibition: Fixed amounts of α -glucosidase were incubated with increasing concentrations of PNPg at 37°C for 15 min, in the absence or presence of inhibitors (concentration equivalent to their IC₅₀). Reactions were terminated, and absorption was measured and analyzed by Lineweaver–Burk plot. All the determinations were performed in triplicate.

Statistical analysis: The data were expressed as the mean \pm SD of three replicates. The analysis was performed using Excel 2013. One-way analysis of variance (ANOVA) and Tukey posttest were used to evaluate the possible differences among the means. *p* values \leq 0.05 were considered as significant differences.

Docking Study: Ligands were constructed in Spartan'10 [14], and their geometry was optimized using MMFF force field. A protein-ligand docking study was carried out based on the crystal structures for C-terminal domain of human intestinal α -glucosidase (PDB: 3TOP) [15], which was retrieved from the Protein Data Bank [16]. Before docking, all of the solvent molecules and the co-crystallized ligand were removed. Molecular docking calculations were performed using Molegro Virtual Docker v. 6.0.1 [17]. A sphere of 12 Å radius was centered in the binding site for searching.

Experimental data indicates palmitic and oleic acids are competitive inhibitors; thus, the active site was chosen as the binding site. Protonation states and assignments of the charges on each protein were based on standard templates of the Molegro Virtual Docker program, and no other charges were necessary to set. Flexible ligand model was used in the docking and subsequent optimization scheme. Different orientations of the ligands were searched and ranked based on their energy scores. The RMSD threshold for multiple cluster poses was set to <1.00 Å. The docking algorithm was set to 5000 maximum iterations with a simplex evolution population size of 100 and a minimum of 50 runs for each ligand. After docking, some further scores were calculated including the binding affinity (MolDock Score) and re-ranking score (Rerank Score). The re-ranking score utilizes a more advanced scoring scheme than that used during docking and is often more useful for accurate ranking of the poses. Poses with lower score were selected for further analysis. To assess the efficacy of this procedure for finding low energy solutions, we docked the co-crystallized ligand (acarbose).

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References

- [1] Petrovska BB. (2012) Historical review of medicinal plants' usage. *Pharmacognosy Review*, **6**, 1–5.
- [2] WHO (World Health Organization) (2011) The World Traditional Medicines Situation, in *Traditional medicines: Global Situation, Issues and Challenges*. Geneva 3, 1–14.
- [3] Magaña A, Gama L, Mariaca R. (2010) El uso de las plantas medicinales en las comunidades chontales de Nacajuca, Tabasco. México. *Polibotánica*, **29**, 213–262.
- [4] Roig, Mesa JT. (1988) *Plantas medicinales aromáticas o venenosas de Cuba*. 2da. Edición. Editorial Científico Técnica. La Habana Cuba. P. 647–648.
- [5] Joly LG, Guerra S, Séptimo R, Solís PN, Correa M, Gupta M, Levy S, Sandberg F. (1987) Ethnobotanical inventory of medicinal plants used by the Guaymí Indians in Western Panama. Part I. *Journal of Ethnopharmacology*, **20**, 145–171.
- [6] Coe FG, Anderson GJ. (2005) Snakebite ethnopharmacopoeia of eastern Nicaragua. *Journal of Ethnopharmacology*, **96**, 303–323.
- [7] Montero JJ. Adjuvant Extract for use against Diabetes and related diseases. PAT:WO2009104944.
- [8] Cherigo L, Salazar M, Martínez-Luis S. (2015) Evaluation of phytotoxic activity of *Blechnum Pyramidatum*, a weed of Panamanian coffee crops. *International Journal of Current Research*, **7**, 24172–24176.
- [9] (a) Chan HH, Sun HD, Reddy MV, Wu TS (2010) Potent α -glucosidase inhibitors from the roots of *Panax japonicus* C. *Phytochemistry*, **71**, 1360–1364; (b) Apostolidis E, Kwon YI, Shetty K. (2007) Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. *Innovative Food Science and Emerging Technologies*, **8**, 46–54; (c) Subramanian R, Asmawi MZ, Sadikun A. (2008) *In vitro* α -glucosidase and α -amylase enzyme inhibitory effects of *Andrographis paniculata* extract and andrographolide. *Acta Biochimica Polonica*, **55**, 391–398.
- [10] Bulama JS, Dangoggo SM, Halilu ME, Tsafe AI, Hassan SW. (2014) Isolation and characterization of palmitic acid from ethyl acetate extract of root bark of *Terminalia glaucescens*. *Chemistry and Materials Research*, **6**, 140–143.
- [11] (a) Artanti N, Tachibana S, Kardono LB, Sukiman H. (2012) Isolation of alpha-glucosidase inhibitors produced by an endophytic fungus, *Colletotrichum* sp. TSC13 from *Taxus sumatrana*. *Pakistan Journal of Biological Sciences*, **15**, 673–679; (b) Miyazawa M, Yagi N, Taguchi K. (2005) Inhibitory Compounds of α -Glucosidase Activity from *Arctium lappa* L. *Journal of Oleo Science*, **54**, 589–594; (c) Su C-H, Hsu C-H, Ng LT. (2013) Inhibitory potential of fatty acids on key enzymes related to type 2 diabetes. *BioFactors*, **39**, 415–421.
- [12] (a) Lopez D, Cherigo L, Spadafora C, Loza-Mejía MA, Martínez-Luis, S. (2015) Phytochemical composition, antiparasitic and α -glucosidase inhibition activities from *Pelliciera rhizophorae*. *Chemical Central Journal*, **9**, 53. (b) Lopez D, Cherigo L, de Seda A, Spadafora C, Martínez-Luis, S. (2018) Evaluation of antiparasitic, anticancer, antimicrobial and hypoglycemic properties of organic extracts from Panamanian mangrove plants. *Asian Pacific Journal of Tropical Medicine*, **11**, 32–39.
- [13] Copeland RA. (2000) *Enzymes: a practical introduction to structure, mechanisms and data analysis*. Wiley-VCH, New York.
- [14] Spartan 10 for Windows, Wavefunction Inc, Irvine, CA.
- [15] Ren L, Qin X, Cao X, Wang L, Bai F, Bai G, Shen Y. (2011) Structural insight into substrate specificity of human intestinal maltase-glucoamylase. *Protein Cell*, **2**, 827–836.
- [16] Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. (2000) The protein data bank. *Nucleic Acids Research*, **28**, 235–242.
- [17] Thomsen R, Christensen MH. (2006) MolDock: a new technique for high-accuracy molecular docking. *Journal of Medicinal Chemistry*, **49**, 3315–3321.