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ARTICLE

Elucidation of inhibitor-binding pocket of D-amino acid oxidase using docking simulation and *N*-sulfanylethylanilide-based labeling technology

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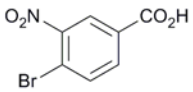
Because of relevance of D-serine (D-Ser) to schizophrenia, inhibitors of D-amino acid oxidase (DAO), which catalyzes degradation of D-Ser in the presence of flavin adeninde dinucleotide (FAD), are expected as anti-schizophrenia therapeutics. In this study, binding pockets of DAO to its inhibitor 4-bromo-3-nitrobenzoic acid were searched by combining *in silico* docking simulation and labeling experiments employing an *N*-sulfanylethylanilide-based labeling technology we have developed. The results clearly demonstrated that there are two binding pockets: one is shared with D-Ser and FAD, and the other is an unexpected cleft between subunits of a DAO dimer. These findings will provide insight to aid the development of new hDAO inhibitors. In addition, it was also proved that our labeling technology could be applicable to elucidate the binding pockets of proteins.

Introduction

D-Amino acid oxidase (DAO), which was first reported by Krebs in 1935,[1] catalyzes oxidative deamination of D-amino acids to generate corresponding imino acids in the presence of flavine adenine dinucleotide (FAD) as a co-factor.[2] D-Serine (Ser) is a substrate of DAO, and its distribution in central nervous system is known to be similar to that of *N*-methyl-D-aspartate receptor (NMDAR).[3] A physiological role of D-Ser is reported to be a neuromodulatory factor that binds to NMDAR together with glutamate to allow for opening of the ion channel.[4] It is suggested that hypofunction of NMDAR is relevant to schizophrenia,[5] and administration of D-Ser to schizophrenia patients significantly improved their symptoms.[6] The expression level and activity of DAO are increased in brain of schizophrenia patients,[7] and DAO degrades D-Ser to suppress the function of NMDAR; therefore, DAO inhibitors are expected as new therapeutics for treatment of schizophrenia.[8,9a-e]



1

Fig. 1 Structure of BNBA **1**.

Several crystal structures of complexes of human DAO (hDAO) and inhibitors of hDAO have been reported.[9] For example, a research group led by Fukui, one of the authors of the present study, first determined the crystal structure of an hDAO-inhibitor complex in which the inhibitor was benzoate (K_i of benzoate to hDAO = 7 μM).[9g] The crystal structures reported so far show that the hDAO inhibitors are bound to a pocket wherein D-Ser would bind for subsequent oxidative deamination. In contrast, some inhibitors that were much larger than the pocket were also reported by Fukui's group.[10] Therefore, clarification of a binding pocket other than the area shared with D-Ser will provide insight to aid the development of new hDAO inhibitors.

We recently established a high-throughput screening system for hDAO inhibitors and found that 4-bromo-3-nitrobenzoic acid (BNBA) **1** depicted in Fig. 1 shows the inhibitory activity (K_i = 2.5 μM).[11] In this paper, computational and experimental approaches for clarification of the binding pocket of hDAO to BNBA **1** are described.

Results and discussion

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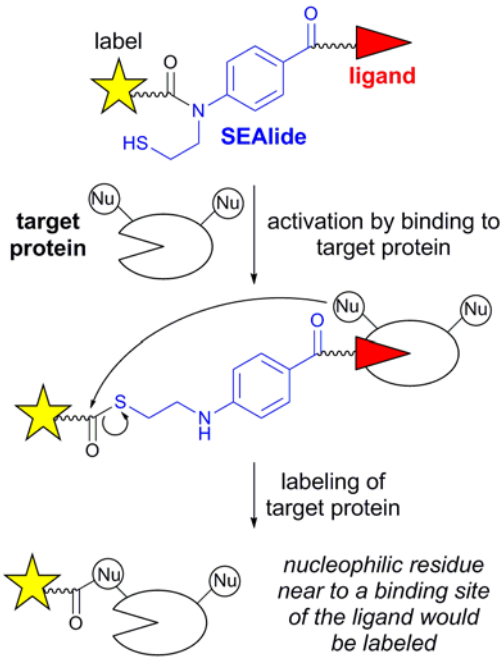
Computational docking study to elucidate a binding pocket of hDAO to BNBA 1

For the computational docking study, the crystal structure of hDAO complex with FAD and benzoate reported by Fukui et al. was employed (PDB ID: 2du8).[9g] In this study, docking simulation of BNBA **1** and hDAO dimer in both the presence and absence of FAD (holo-form and apo-form, respectively) was carried out, because dissociation of FAD and hDAO should be taken into account (K_d = 8 ± 2 μM).[12,13] First, the docking study without FAD was examined. After removal of small molecules followed by addition of polar hydrogens and Gasteiger charges using Autodock Tools,[14] an hDAO dimer was extracted from the crystal structure.[15] A docking study of the obtained hDAO dimer with BNBA **1** using AutoDock Vina[16] predicted that BNBA **1** is bound in an FAD-D-Ser-binding pocket (Fig. 2A). The docking study in the presence of FAD was also conducted similarly to that without FAD, and the results are shown in Fig. 2B. In this case, presence of a new binding pocket between hDAO subunits was indicated. These results clearly suggested that there are two binding pockets: the FAD-D-Ser-binding pocket and the cleft between hDAO subunits. The former pocket is easily acceptable, because many hDAO inhibitors are bound in this pocket;[9] however, the later cleft was unexpected. Therefore, we next attempted to demonstrate the two binding sites experimentally.

Fig. 2 (page 8)

Experimental labeling study of hDAO to determine the binding pocket of 1

The binding pocket predicted by the computational study was experimentally evaluated using a protein labeling technology. An affinity labeling such as photo-affinity labeling[17] or ligand-directed labeling[18,19] is widely employed to determine a binding pocket of a ligand, because it enables introduction of the label moiety to an amino acid near to the binding pocket and the labeled amino acid can be easily identified using proteomics protocols. We recently reported a new ligand-directed labeling technology termed SEAL (*N*-sulfanylethylanilide-based labeling), and its labeling mechanism is shown in Scheme 1.[20] After binding of a SEAL reagent consisting of a label, *N*-sulfanylethylanilide (SEAlide),[21,22] and a ligand to its target protein, an amide bond of the SEAL reagent will be activated to a thioester via N–S acyl transfer. A nucleophilic group near to the binding pocket will then attack to the thioester to generate a labeled target protein. As introduction of the label to a lysine residue near to the binding pocket was observed in the previous study, in which a human carbonic anhydrase I and benzenesulfonamide pair was employed,[20] we applied the SEAL technology to clarify the binding pocket of hDAO to BNBA **1**.



Scheme 1 Ligand-directed labeling of a target protein utilizing SEAL technology (Nu: nucleophilic functional group).

It is known that benzoate also acts as an inhibitor of hDAO.[9g] Therefore, a carboxylic acid moiety of **1** was retained and an electron withdrawing bromine away from the carboxylic acid was replaced by an electron withdrawing amide (σ_{para}: Br = 0.23; CONHMe = 0.36)[23] for introduction of the SEAL unit (Fig. 3). In this study, SEAL reagent **2** possessing biotin as the label was designed. Preparation of the ligand moiety of SEAL reagent **2** is shown in Scheme 2. Starting from **4**,[24] protected diethylamine linker moiety **5**[25] was introduced and the allyloxycarbonyl (Alloc) group was removed in the presence of Pd(0) to generate amine **7**. Conjugation of ligand moiety to the SEAlide unit was next examined (Scheme 3). Following removal of a fluorenylmethoxycarbonyl (Fmoc) group of protected glycyl SEAlide **8**,[22c] the generated amine was coupled with biotin and an allyl group of **9** was removed in the presence of Pd(0). Carboxylic acid **10** was then coupled with ligand derivative **7**, and the generated conjugate **11** was subjected to deprotection under acidic conditions to afford SEAL reagent **2**. Negative control **3** without the ligand moiety was also synthesized similarly to the SEAL reagent **2** (Fig. 3 and Scheme 4).

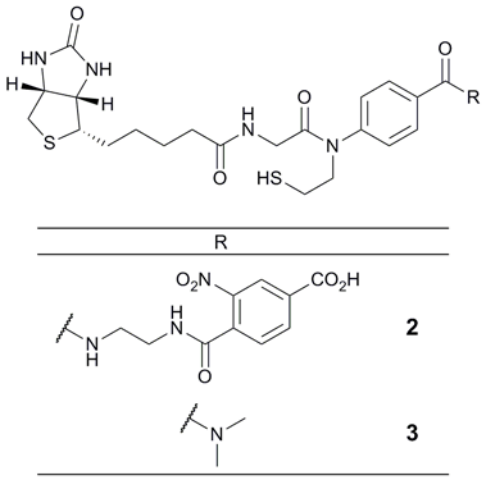
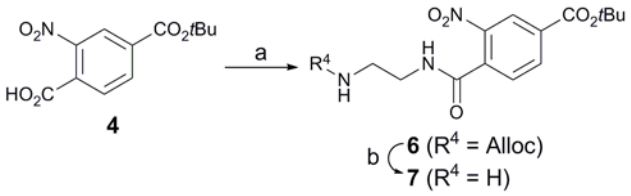
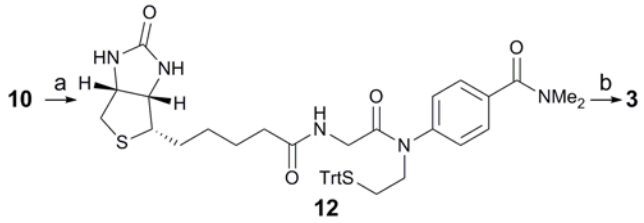


Fig. 3 Structure of SEAL reagent **2** and its negative control **3**.



Scheme 2 Synthesis of ligand derivative for preparation of SEAL **2**. a) $H_2NCH_2CH_2NHAlloc$ (**5**), EDC·HCl, DMAP, CH_2Cl_2, 61%; b) $Pd(PPh_3)_4$, *N*-methylaniline, THF, 62%.

Scheme 3 (page 9)



Scheme 4 Synthesis of negative control **3**. a) dimethylamine hydrochloride, EDC·HCl, DMAP, CH_2Cl_2, 71%; b) TFA, triisopropylsilane, CH_2Cl_2, 66%.

With SEAL reagent **2** and negative control **3** in hand, we next examined labeling of hDAO with the SEAL reagent (Fig. 4). In this study, hDAO with a His tag sequence at an *N*-terminal position was expressed,[9b] purified, and stored in a solution containing stabilizers (FAD, dithiothreitol, sodium benzoate, and KCl) until subsequent labeling experiments. The stock solution of hDAO was then diluted with Tris buffer (50 mM, pH 8.3), and the resulting solution was treated with SEAL reagent **2** at 37 °C for 24 or 48 h (final concentration: 1, 5, or 10 μM SEAL reagent **2**, 10 μM hDAO, 1 μM FAD, 10 μM DTT, 20 μM sodium benzoate, 5 mM KCl). The resulting mixture was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and a biotinylated hDAO was visualized by Western blotting using a streptavidin-horseradish peroxidase conjugate (SAv-HRP). Whereas the co-existing benzoate in the labeling reaction mixture would compete with BNBA, efficient labeling of hDAO with SEAL **2** occurred when 5 and above micromolar **2** was employed (Fig. 4A).[26] Next, confirmation was made as to whether the labeling was ligand-directed. Competitive inhibition assay was first examined and the labeling by SEAL reagent **2** was hampered by addition of 10 equivalent of BNBA **1** (Fig. 4B). When negative control **3** without the ligand unit was employed instead of SEAL reagent **2**, almost no labeling was observed even if 100 μM of **3** was employed (Fig. 4C). These results suggest that the biotinylation of hDAO using SEAL reagent **2** proceeded in a ligand-directed manner.

Next, determination of the labeled residue of hDAO was carried out. Mixture of biotinylated hDAO obtained after labeling with 5 μM of **2** for 48 h followed by SDS-PAGE was subjected to in-gel proteolytic digestion using trypsin, and resulting peptide fragments were extracted from the gel and analyzed by nanoLC-MS/MS. Only two of 12 lysine (Lys) residues were identified as the labeled residues (Fig. S2 in ESI), and they are depicted in Fig. 5. The Lys163 found at an entrance of the pocket for FAD and D-Ser was labeled as we expected (Fig. 5A). Furthermore, labeling of the Lys204 located near to the new binding pocket predicted by *in silico* study was also observed (Fig. 5B). The computational and experimental results both provided strong evidence that the two binding pockets for inhibitor **1** exist on hDAO.

Finally, *in silico* docking study of the SEAL reagent to an hDAO dimer was conducted to confirm whether a pose responsible for the ligand-directed labeling could be obtained.

Fig. 4 (page 9)

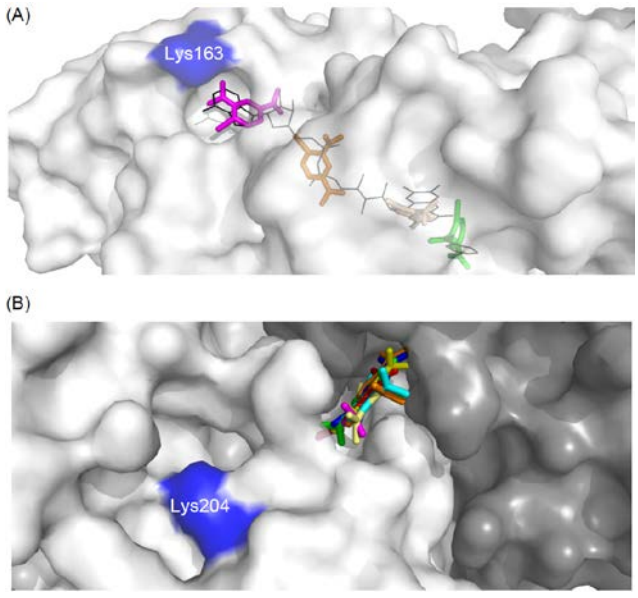


Fig. 5 Labeled Lys163 and Lys204 of hDAO. Structures of (A) and (B) correspond to that in Fig. 2. Lys163 in (A) and Lys204 in (B) are colored in blue.

In this study, thioester **13**, in which the biotinylglycine moiety of SEAL reagent **2** was replaced by an acetyl group for simplification, was employed as a model of the activated SEAL reagent (Fig. 6A). After the docking studies in the absence and presence of FAD, poses in which the ligand unit was in the predicted binding pocket and a carbonyl carbon of the thioester unit was located near to the labeled lysine were observed within the top nine stable poses in each case (Fig. 6B and C).[27] These results rationalize that labeling of Lys163 and 204 by SEAL reagent **2** would occur via binding of the ligand moiety to the pockets that were clarified by the computational and experimental studies.

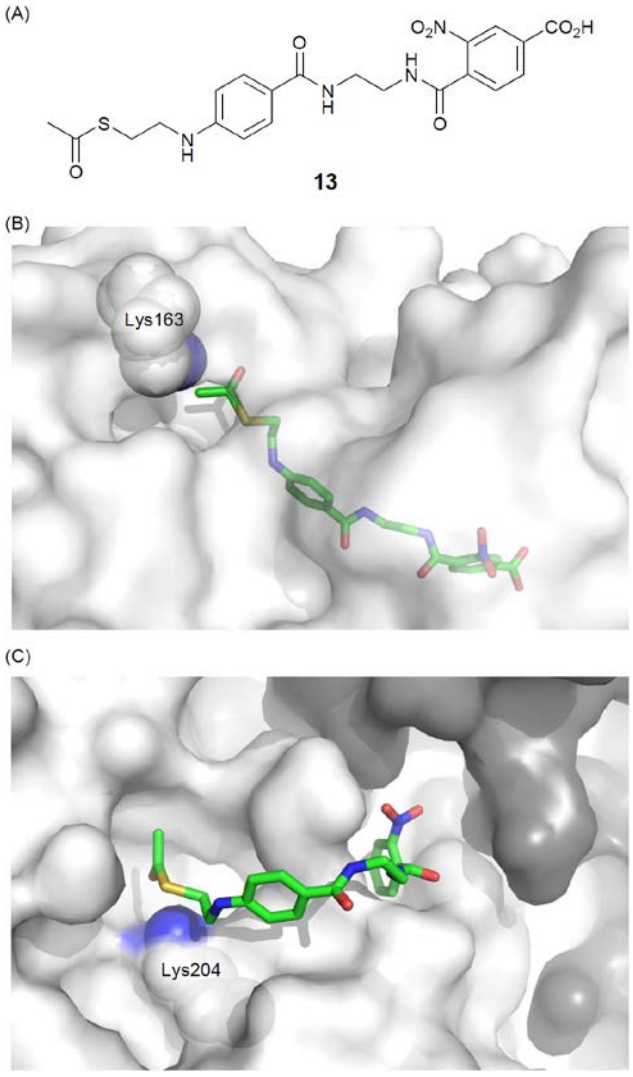


Fig. 6 Docking study of the SEAL reagent **13** to hDAO. (A) Structure of a model of the activated SEAL reagent that was used for the docking study; (B and C) Structures of hDAO dimer-**13** complexes predicted by the docking study in the absence (A) or presence (B) of FAD. A side chain of Lys163 in (B) and Lys204 in (C) are depicted as space-filling models (C: white; N: blue). SEAL reagent **13** is shown in a stick model (C: green; N: blue; O: red; S: yellow).

Conclusions

Using computational docking simulation and experimental SEAL technology, we demonstrated that hDAO has two binding pockets of its inhibitor BNBA **1**. One pocket is shared with FAD and D-Ser, and the other is an unexpected new pocket between hDAO subunits. The former pocket could be responsible for inhibitory activity of BNBA, because D-Ser and other inhibitors are known to be bound in this pocket; however, it is unclear at present whether the later new pocket shares responsibility for the inhibitory activity. Elucidation of the role of the new binding pocket and its ligand-induced events employing, for instance, mutant or chemically modified hDAO should be examined in due course.

Experimental section

Computational study

MM2 optimized 3D structures of BNBA **1** and SEAL reagent **13** were obtained using ChemBio3D Ultra (version 14.0.0.117, CambridgeSoft). A structure of an hDAO dimer was generated from a crystal structure (PDB ID: 2du8).[9g] AutoDock Tools 1.5.6 was employed for removal of unnecessary small molecules, extraction of an hDAO dimer, and addition of polar hydrogens and Gasteiger charges.[14] Docking studies of the obtained hDAO dimer were carried out using AutoDock Vina 1.1.2.[16] The GRID box parameters used are as follow: center X, center Y, center Z, dimension, exhaustiveness = 45.713, 50.082, 13.005, 60 × 110 × 76 Å, 4000. The obtained results shown in Figure 2, 5, and 6 (B and C) were visualized using PyMOL (The PyMOL Molecular Graphics System, Version 1.7.0.1 Schrödinger, LLC.). In the case of docking study of **13** to hDAO in the absence of FAD, a side chain of Lys163 was treated as flexible.[27]

Experimental study

General procedures

All chemical reactions were carried out under a positive presser of argon at room temperature unless otherwise noted. For column chromatography, silica gel 60 N (spherical, neutral, Kanto Chemical Co. Inc.) was employed. Thin layer chromatography was performed on precoated plates (0.25 nm, silica gel Merck Kieselgel 60F245). NMR spectra were recorded using a Bruker AV400N at 400 MHz frequency for ^{1}H and a JEOL JNM-AL300 at 75 MHz frequency for ^{13}C. Chemical shifts are calibrated to the solvent signal. A Waters MICROMASS® LCT PREMIER (ESI-TOF) was employed for measurement of mass spectra. Optical rotations and IR spectra were measured using a JASCO P-2200 polarimeter (concentration in g/100 mL) and a JASCO FT-IR 6200, respectively. For CBB staining, CBB Staining One (Nacalai tesque) was employed.

Synthesis of the SEAL reagents

***tert*-Butyl 4-[(2-{[(allyloxy)carbonyl]amino}ethyl)carbamoyl]-3-nitrobenzoate (6):** To carboxylic acid **4**[24] (88 mg, 0.33 mmol) in CH_2Cl_2 (2 mL) were added amine **5**[25] (52 mg, 0.36 mmol),

EDC·HCl (69 mg, 0.36 mmol), and DMAP (4.0 mg, 0.033 mmol), and the obtained mixture was stirred for 10 h. After addition of H_2O, the mixture was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The product was purified by column chromatography ($CHCl_3$/MeOH = 10/0 to 20/1 (v/v)) and 79 mg of *tert*-butyl ester **6** (0.20 mmol, 61%) was obtained as a white solid: ^{1}H NMR ($CDCl_3$) δ = 1.60 (9H, s), 3.39 (2H, br q, *J* = 5.2 Hz), 3.51 (2H, dt, *J* = 5.8 and 5.2 Hz), 4.44 (2H, dt, *J* = 5.6 and 1.4 Hz), 5.16 (1H, ddt, *J* = 10.4, 2.7 and 1.4 Hz), 5.24 (1H, ddt, *J* = 17.2, 2.7 and 1.4 Hz), 5.51 (1H, br t, *J* = 5.8 Hz), 5.83 (1H, ddd, *J* = 17.2, 10.4 and 5.6 Hz), 7.15 (1H, br t, *J* = 5.2 Hz), 7.51 (1H, d, *J* = 7.8 Hz), 8.17 (1H, dd, *J* = 7.8 and 1.5 Hz), 8.49 (1H, d, *J* = 1.5 Hz); ^{13}C NMR ($CDCl_3$) δ = 28.2, 40.3, 41.1, 65.9, 83.2, 117.9, 125.4, 129.0, 132.7, 134.3, 134.5, 135.8, 146.5, 157.5, 162.9, 166.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{18}H_{23}N_3NaO_7$ ([M + Na]$^+$) 416.1434, found 416.1441; IR (KBr) 785, 851, 922, 999, 1177, 1570, 1664, 1733, 2927, 3284, 3663 cm^{-1}.

***tert*-Butyl 4-[(2-aminoethyl)carbamoyl]-3-nitrobenzoate (7):** A solution of alloc derivative **6** (69 mg, 0.18 mmol), $Pd(PPh_3)_4$ (10 mg, 9.0 µmol), and *N*-methylaniline (0.20 µL, 1.8 mmol) in THF (2 mL) was stirred for 10 h. After concentration in vacuo, the obtained residue was purified by column chromatography ($CHCl_3$/MeOH = 10/0 to 10/1 (v/v)) and 34 mg of carboxylic acid **7** (0.11 mmol, 62%) was obtained as a white solid: ^{1}H NMR ($CDCl_3$) δ = 1.60 (9H, s), 2.52 (2H, br s), 2.93 (2H, br t, *J* = 5.3 Hz), 3.45 (2H, br s), 7.29 (1H, br s), 7.53 (1H, d, *J* = 7.8 Hz), 8.17 (1H, dd, *J* = 7.8 and 1.4 Hz), 8.50 (1H, d, *J* = 1.4 Hz); ^{13}C NMR ($CDCl_3$) δ = 28.2, 40.6, 42.0, 83.2, 125.4, 129.1, 134.3, 134.4, 136.0, 146.5, 162.9, 166.5; HRMS (ESI-TOF) *m*/*z* calcd for $C_{14}H_{19}N_3NaO_5$ ([M + Na]$^+$) 332.1222, found 332.1229; IR (KBr) 766, 843, 923, 990, 1121, 1151, 1308, 1357, 1548, 1648, 1688, 1721, 2986, 3322 cm^{-1}.

Allyl 4-[2-(5-{(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl}pentanamido)-*N*-(2-{tritylthio}ethyl)acetamido]benzoate (9): Fmoc derivative **8**[22c] (3.2 g, 4.2 mmol) in $CHCl_3$ (20 mL) was treated with piperidine (5 mL, 51 mmol) for 1 h. Following to concentration of the reaction mixture in vacuo, the obtained residue was dissolved in DMF (18 mL), and EDC·HCl (0.88 mg, 4.6 mmol), DMAP (51 mg, 0.42 mmol), and D-biotin (1.1 g, 4.6 mmol) were added to the solution. After 8 h of stirring followed by addition of H_2O, the reaction mixture was extracted with EtOAc. The obtained organic layer was washed with 5% (v/v) $KHSO_4$ aq. follwed by brine, dried over Na_2SO_4, and concentrated in vacuo. The product was purified by column chromatography ($CHCl_3$/MeOH = 10/0 to 10/1 (v/v)) and 2.4 g of biotinylated product **9** (3.1 mmol, 74%) was obtained as a yellow solid: $[\alpha]^{22}_D$ +20.4 (*c* 1.00, $CHCl_3$): ^{1}H NMR ($CDCl_3$) δ = 1.31 (2H, quint, *J* = 7.3 Hz), 1.48-1.68 (4H, m), 2.15 (2H, t, *J* = 7.4 Hz), 2.30 (2H, t, *J* = 7.4 Hz), 2.63 (1H, d, *J* = 12.8 Hz), 2.75 (1H, dd, *J* = 12.8 and 4.8 Hz), 3.00 (1H, dt, *J* = 7.1 and 4.5 Hz), 3.27-3.42 (2H, m), 3.46 (1H, d, *J* = 17.0 Hz), 3.55 (1H, d, *J* = 17.0 Hz), 4.17 (1H, dd, *J* = 7.6 and 4.5 Hz), 4.35 (1H, dd, *J* = 7.6 and 4.8 Hz), 4.78 (2H, dt, *J* = 5.7 and 1.3 Hz), 5.23 (1H, dtd, *J* = 10.4, 2.7 and 1.3 Hz), 5.35 (1H, dtd, *J* = 17.1, 2.7 and 1.3 Hz), 5.97 (1H, ddt, *J* = 17.1, 10.4 and 5.7 Hz), 6.99 (2H, d, *J* = 8.4 Hz), 7.02-7.14 (9H, m), 7.18-7.26 (6H, m), 7.28 (1H, br s), 7.95 (2H, d, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ = 25.6, 28.0, 28.3, 29.3, 35.6, 40.5, 41.9, 48.7, 55.8, 60.4, 61.8, 65.9, 67.0, 118.6, 126.7, 127.9, 128.2, 129.5, 130.2, 131.4, 132.0, 144.2, 144.4, 164.6, 165.2, 168.8, 173.6; HRMS (ESI-TOF) *m*/*z* calcd for $C_{43}H_{46}N_4NaO_5S_2$ ([M + Na]$^+$) 785.2807, found 785.2806; IR (KBr) 701, 747, 854, 934, 1016, 1117, 1168, 1271, 1443, 1603, 1664, 1708, 3317 cm^{-1}.

4-[2-(5-{(3a*S*,4*S*,6a*R*)-2-Oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl}pentanamido)-*N*-(2-{tritylthio}ethyl)acetamido]benzoic acid (10): Starting from **9** (2.4 g, 3.1 mmol), carboxylic acid **10** (2.1 g, 2.9 mmol, 94%, a yellow solid) was synthesized according to the synthesis of **7**: $[\alpha]^{22}_D$ +29.1 (*c* 1.00, $CHCl_3$): ^{1}H NMR ($CDCl_3$) δ = 1.42 (2H, br quint, *J* = 6.4 Hz), 1.48-1.80 (4H,br m), 2.25 (2H, br t, *J* = 6.8 Hz), 2.37 (2H, br t, *J* = 6.8 Hz), 2.73 (1H, d, *J* = 12.8 Hz), 2.82 (1H, br dd, *J* = 12.8 and 4.4 Hz), 3.11 (1H, br m), 3.45 (2H, br t, *J* = 6.8 Hz), 3.60 (1H, d, *J* = 18.0 Hz), 3.68 (1H, d, *J* = 18.0 Hz), 4.28 (1H, br dd, *J* = 7.2 and 4.4 Hz), 4.44 (1H, br t, *J* = 4.4 Hz), 7.02 (2H, br d, *J* = 7.9 Hz), 7.08-7.20 (15H, m), 7.29-7.33 (1H, m), 7.31 (6H, d, *J* = 7.1 Hz), 7.97 (2H, br d, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$) δ = 24.6, 25.3, 25.6, 28.2, 29.4, 40.7, 42.2, 55.5, 55.6, 60.6, 62.1, 67.1, 126.9, 128.0, 128.1, 128.1, 129.6, 131.7, 143.8, 144.5, 164.9, 168.3, 168.5, 173.8; HRMS (ESI-TOF) *m*/*z* calcd for $C_{40}H_{42}N_4NaO_5S_2$ ([M + Na]$^+$) 745.2494, found 745.2496; IR (KBr) 703, 750, 1118, 1169, 1269, 1443, 1662, 1676, 1703, 3309 cm^{-1}.

***tert*-Butyl 3-nitro-4-[(2-{4-[2-(5-{(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl}pentanamido)-*N*-(2-{tritylthio}ethyl)acetamido]benzamido}ethyl)carbamoyl]benzoate (11):** Carboxylic acid **10** (120 mg, 0.17 mmol) and amine **7** (53 mg, 0.17 mmol) were coupled in the presence of EDC·HCl (36 mg, 0.19 mmol) and DMAP (2.1 mg, 17 µmol) in CH_2Cl_2 (2 mL) for 5 h. After addition of H_2O followed by extraction with EtOAc, and the obtained organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The product was purified by column chromatography ($CHCl_3$/MeOH = 10/0 to 10/1 (v/v)) and 110 mg of conjugate **11** (0.11 mmol, 62%) was obtained as a white solid: $[\alpha]^{18}_D$ +23.3 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ = 1.24-1.36 (2H, m), 1.45-1.57 (4H, m), 1.60 (9H, s), 2.08 (2H, br t, *J* = 7.8 Hz), 2.35 (2H, t, *J* = 7.2 Hz), 2.65 (1H, d, *J* = 12.7 Hz), 2.82 (1H, dd, *J* = 12.7 and 4.8 Hz), 3.04 (1H, dd, *J* = 11.2 and 6.9 Hz), 3.34-3.49 (4H, m), 3.50-3.70 (4H, m), 4.20 (1H, dd, *J* = 6.9 and 4.5 Hz), 4.38 (1H, dd, *J* = 4.8 and 4.5 Hz), 5.78 (1H, br s), 6.62 (1H, br s), 6.98 (2H, d, *J* = 8.4 Hz), 7.12-7.23 (10H, m), 7.31 (6H, d, *J* = 6.9 Hz), 7.55 (1H, d, *J* = 8.0 Hz), 7.75 (2H, d, *J* = 8.1 Hz), 7.86 (1H, br s), 7.95 (1H, br s), 8.18 (1H, dd, *J* = 8.0 and 1.5 Hz), 8.50 (1H, d, *J* = 1.5 Hz); ^{13}C NMR ($CDCl_3$) δ = 24.6, 25.3, 25.5, 28.2, 29.8, 32.8, 40.5, 40.6, 40.8, 48.7, 55.5, 60.6, 62.0, 67.1, 70.7, 83.1, 125.3, 126.9, 128.0, 128.2, 129.3, 129.6, 134.4, 136.0, 142.6, 144.5, 146.7, 163.0, 166.8, 171.5, 173.8; HRMS (ESI-TOF) *m*/*z* calcd for $C_{54}H_{59}N_7NaO_9S_2$ ([M + Na]$^+$) 1036.3713, found 1036.3737; IR (KBr) 700, 746, 848, 927,

1017, 1034, 1121, 1153, 1171, 1253, 1298, 1444, 1500, 1539, 1605, 1659, 1681, 1692, 1714, 2926, 3308 cm^{-1}.

4-[(2-{4-[*N*-(2-Mercaptoethyl)-2-(5-{(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl}pentanamido)acetamido]benzamido}ethyl)carbamoyl]-3-nitrobenzoic acid (2): To protected derivative **11** (110 mg, 0.11 mmol) in CH_2Cl_2 (2 mL) were added triisopropylsilane (25 µL, 0.12 mmol) and TFA (84 µL, 1.1 mmol), and the obtained mixture was stirred for 2 h. After concentration of the reaction mixture in vacuo, the residue was purified by column chromatography ($CHCl_3$/MeOH = 10/1 to 1/1 (v/v)) and 62 mg of SEAL reagent **2** (87 µmol, 79%) was obtained as a white solid: $[\alpha]^{20}_D$ +18.3 (*c* 1.00, DMSO); ^{1}H NMR (methanol-d_4) δ = 1.36-1.80 (6H, m), 2.18-2.35 (2H, m), 2.65 (2H, t, *J* = 7.4 Hz), 2.69 (1H, d, *J* = 12.8 Hz), 2.92 (1H, dd, *J* =12.8 and 4.8 Hz), 3.20 (1H, ddd, *J* = 7.9, 6.7 and 4.4 Hz), 3.58-3.70 (6H, m), 3.88 (2H, br t, *J* = 7.4 Hz), 4.31 (1H, dd, *J* = 7.8 and 4.4 Hz), 4.49 (1H, dd, *J* = 7.8 and 4.8 Hz), 7.51 (br d, *J* = 8.4 Hz), 7.72 (1H, d, *J* = 7.8 Hz), 7.97 (2H, d, *J* = 8.4 Hz), 8.35 (1H, dd, *J* = 7.8 and 1.4 Hz), 8.62 (1H, d, *J* = 1.4 Hz); ^{13}C NMR (methanol-d_4) δ = 22.5, 26.6, 29.3, 29.4, 36.3, 40.6, 40.8, 41.0, 43.0, 53.8, 56.9, 61.7, 63.2, 126.4, 129.6, 130.2, 130.7, 134.7, 135.6, 137.3, 145.0, 148.0, 166.7, 169.0, 169.4, 170.4, 176.4; HRMS (ESI-TOF) *m/z* calcd for $C_{31}H_{37}N_7NaO_9S_2$ ($[M + H]^+$) 738.1992, found 738.1966; IR (KBr) 1020, 1041, 1124, 1173, 1265, 1349, 1407, 1430, 1535, 1606, 1670, 1687, 2463, 2935, 3261 cm^{-1}.

***N*,*N*-Dimethyl-4-[2-(5-{(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl}pentanamido)-*N*-(2-{tritylthio}ethyl)acetamido]benzamide (12):** Dimethylamine hydrochloride (17 mg, 0.21 mmol) was coupled with carboxylic acid **10** (100 mg, 0.14 mmol) as similar to coupling of **10** with **7**, and the dimethylamide **12** (78 mg, 0.10 mmol, 71%) was obtained as a white solid: $[\alpha]^{18}_D$ +19.9 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ = 1.40 (2H, quint, *J* = 7.3 Hz), 1.58-1.80 (4H, m), 2.16-2.30 (2H, m), 2.37 (2H, t, *J* = 7.2 Hz), 2.73 (1H, d, *J* = 12.7 Hz), 2.85 (1H, dd, *J* = 12.7 and 4.8 Hz), 2.98 (3H, br s), 3.07-3.12 (1H, m), 3.12 (3H, br s), 3.48 (2H, t, *J* = 7.2 Hz), 3.54 (1H, d, *J* = 17.5 Hz), 3.67 (1H, d, *J* = 17.5 Hz), 4.27 (1H, dd, *J* = 7.6 and 4.6 Hz), 4.43 (1H, dd, *J* = 7.6 and 4.8 Hz), 7.03 (2H, d, *J* = 8.4 Hz), 7.13-7.24 (9H, m), 7.31-7.35 (6H, m), 7.41 (2H, d, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ = 25.7, 28.1, 28.3, 29.5, 35.7, 35.7, 40.5, 41.9, 41.9, 48.7, 55.8, 60.5, 61.9, 67.0, 126.8, 128.0, 128.3, 128.9, 129.5, 141.0, 144.5, 164.5, 168.9, 170.3, 173.7; HRMS (ESI-TOF) *m/z* calcd for $C_{42}H_{47}N_5NaO_4S_2$ ($[M + Na]^+$) 772.2967, found 772.2987; IR (KBr) 703, 750, 853, 1083, 1265, 1399, 1444, 1491, 1615, 1659, 1701, 2926, 3302 cm^{-1}.

4-[*N*-(2-Mercaptoethyl)-2-(5-{(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl}pentanamido)acetamido]-*N*,*N*-dimethylbenzamide (3): Deprotection of **3** was performed as similar to that of **11**. Starting from protected derivative **12** (65 mg, 87 µmol), negative control **3** (29 mg, 57 µmol, 66%) was obtained as a white solid: $[\alpha]^{19}_D$ +23.1 (*c* 1.00, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.39-1.48 (3H, m), 1.58-1.80 (4H, m), 2.20-2.33 (2H, m), 2.64 (2H, q, *J* = 6.0 Hz), 2.78 (1H, d, *J* = 12.6 Hz), 2.92 (1H, br d, *J* = 12.6 Hz), 3.02 (3H, br s), 3.13 (3H, br s), 3.72 (2H, br s), 3.86 (2H, br t, *J* = 6.0 Hz), 4.36 (1H, br s), 4.56 (1H, br s), 7.16 (1H, br s), 7.33 (2H, br d, *J* = 7.4 Hz), 7.52 (2H, d, *J* = 7.4 Hz); ^{13}C NMR ($CDCl_3$) δ = 22.1, 25.6, 28.0, 28.1, 35.5, 35.6, 39.8, 40.5, 42.2, 52.6, 55.6, 60.8, 62.1, 128.5, 129.3, 137.1, 141.3, 164.4, 169.3, 170.4, 173.8; HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{33}N_5NaO_4S_2$ ($[M + Na]^+$) 530.1872, found 530.1852; IR (KBr) 723, 753, 799, 834, 854, 1206, 1270, 1410, 1454, 1492, 1604, 1626, 1688, 2530, 2928, 3304 cm^{-1}.

Preparation of hDAO

Following to expression of hDAO with N-terminal His tag in BL21 (DE3) *E. coli* according to the literature,[9b] a modified purification method was employed to obtain highly pure hDAO. Briefly, the expressed hDAO was purified by Ni-NTA chromatography (Ni-NTA Agarose, QIAGEN) followed by anion exchange (HiPrep DEAE 16/60, GE Healthcare) and subsequent gel filtration (Superdex 200, GE Healthcare). The obtained hDAO was stocked in 40 mM Tris buffer (pH 8.3) with stabilizers (100 µM hDAO, 10 µM FAD, 100 µM dithiothreitol, 200 µM sodium benzoate, 50 mM KCl) until subsequent labeling experiments.

Labeling experiment

Typical procedure: To the stock solution of hDAO with stabilizers (4 µL) were added Tris buffer (50 mM, pH 8.3, 34 µL) and a solution of SEAL reagent **2** in DMF (100 µM, 2 µL), and the obtained mixture was incubated at 37 °C for 48 h (final concentration: 10 µM hDAO, 5 µM SEAL reagent **2**, 1 µM FAD, 10 µM dithiothreitol, 20 µM sodium benzoate, 5 mM KCl). After ultrafiltration of the mixture (14,000g, 15 min) followed by addition of 2 × SDS sample loading buffer (40 µL) to the obtained filtrate (80 µL), the resultant was heated at 100 °C for 5 min and then analyzed using SDS-PAGE (12% polyacrylamide). For the Western blotting of the biotinylated proteins, the proteins were transferred to Amersham Hybond-P PVDF Membrane (GE Healthcare) and detected with SAv-HRP and ECL Western Blotting Detection System (GE Healthcare). A chemiluminescent signal was detected with LAS-4000mini (Fujifilm).

Determination of labeled residues

hDAO was labeled with 5 µM of SEAL reagent **2** for 48 h according to the above mentioned method. After SDS-PAGE followed by CBB staining, the gel containing the labeled hDAO was incubated in Tris buffer (50 mM, pH 8.0) with trypsin at 37 °C for 20 h. The digested products were analyzed on nanoLC-MS/MS (LC: UltiMate 3000 RSLCnano system; MS: Orbitrap Elite; Column: Acclaim PepMap RSLC Nano Column (75 µm × 15 cm) (Thermo Fisher Scientific Inc.)). A solvent system consisting of 0.1% aqueous formic acid and 80% aqueous acetonitrile with 0.08% formic acid was employed.

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26 Our following labeling experiments required incubation at 37 °C for 48 h; therefore, benzoate was not removed to expect prevention of denaturing of hDAO during the labeling experiments. Benzoate might affect the labeling of hDAO with SEAL **2**. However, one of BNBA-biding pocket clarified in

the following sections was identical to a binding pocket of benzoate/FAD. We therefore speculated that benzoate, its K_d value to hDAO is not so low (8 ± 2 μM),[12] might compete with SEAL **2** but did not completely block the binding of the SEAL to hDAO.

27 In the case of docking study of **13** without FAD, a side chain of Lys163 was treated as flexible because it was directed away from the binding pocket in the crystal structure.

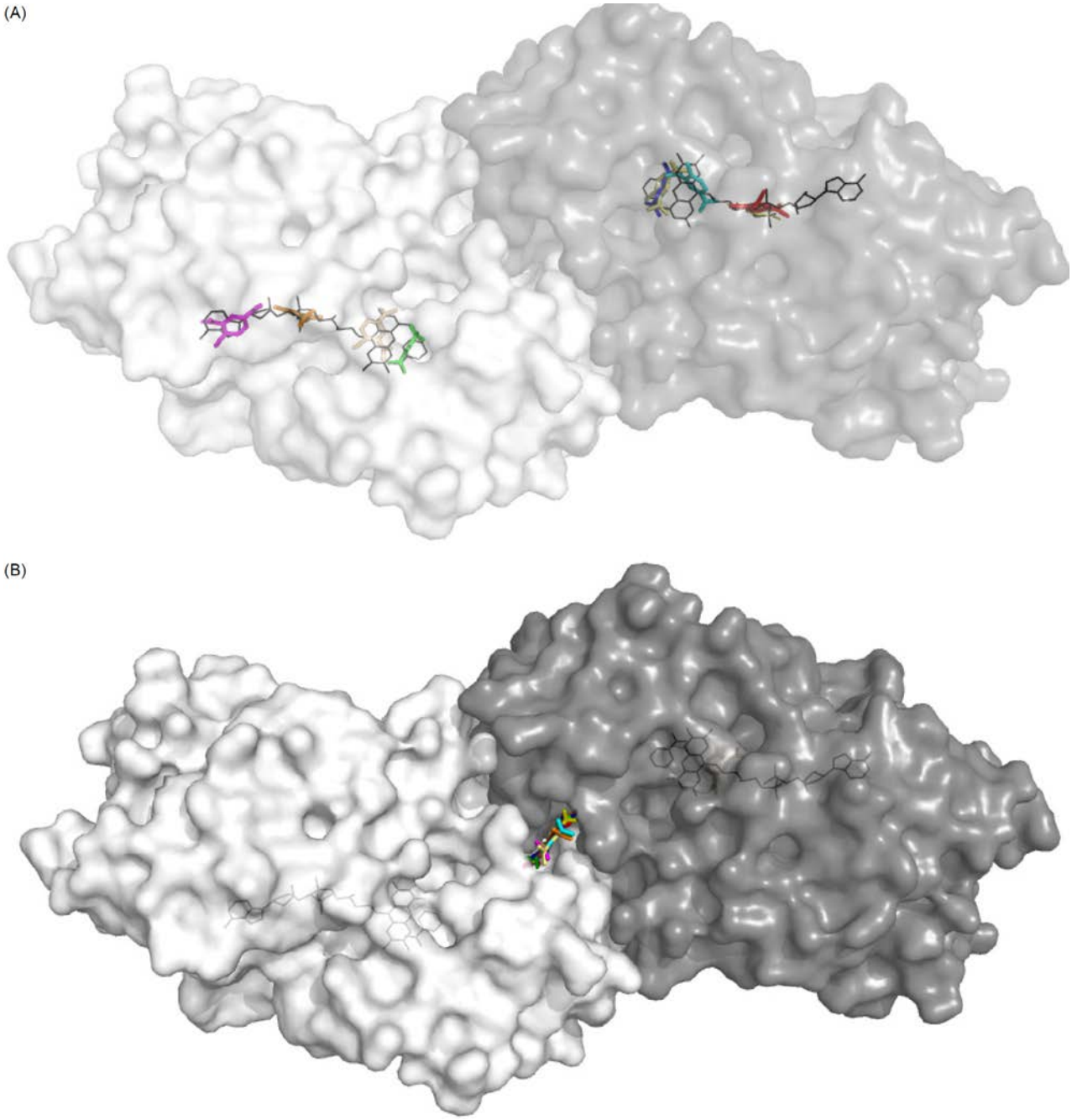
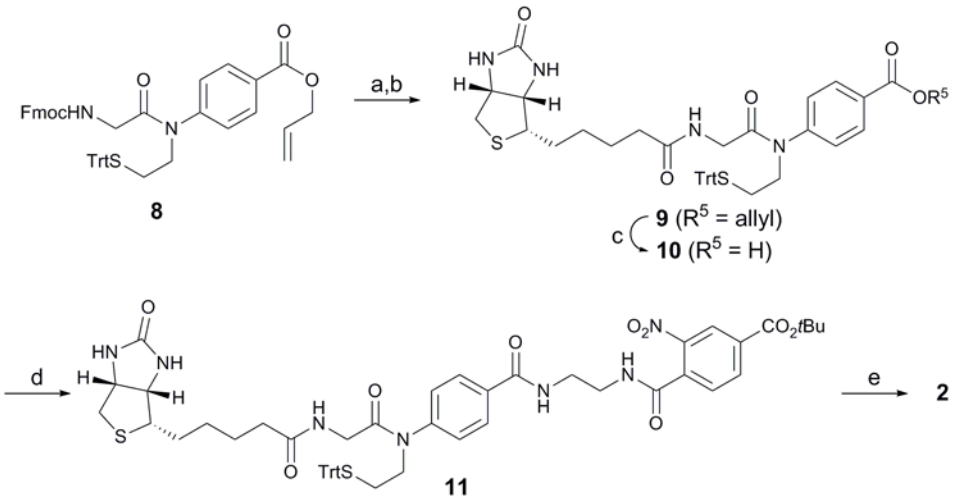


Fig. 2 Structures of hDAO dimer/**1** complexes predicted by the docking study in the absence (A) or presence (B) of FAD. In each figure, nine stable poses predicted by the docking simulation are superimposed. hDAO dimer: white and gray surfaces; FAD and benzoate observed in an original crystal structure: black lines (FAD and benzoate (A) or benzoate (B) were removed before the docking study); nine predicted poses of BNBA **1**: other color sticks. PyMol session files of Fig. 2A and 2B can be found in ESI (Fig_2A.pse and Fig_2B.pse, respectively).



Scheme 3 Synthesis of SEAL **2**. a) piperidine, $CHCl_3$; b) D-biotin, EDC·HCl, DMAP, DMF, 74% (2 steps); c) $Pd(PPh_3)_4$, *N*-methylaniline, THF, 94%; d) **7**, EDC·HCl, DMAP, CH_2Cl_2, 62%; e) TFA, iPr_3SiH, CH_2Cl_2, 79%.

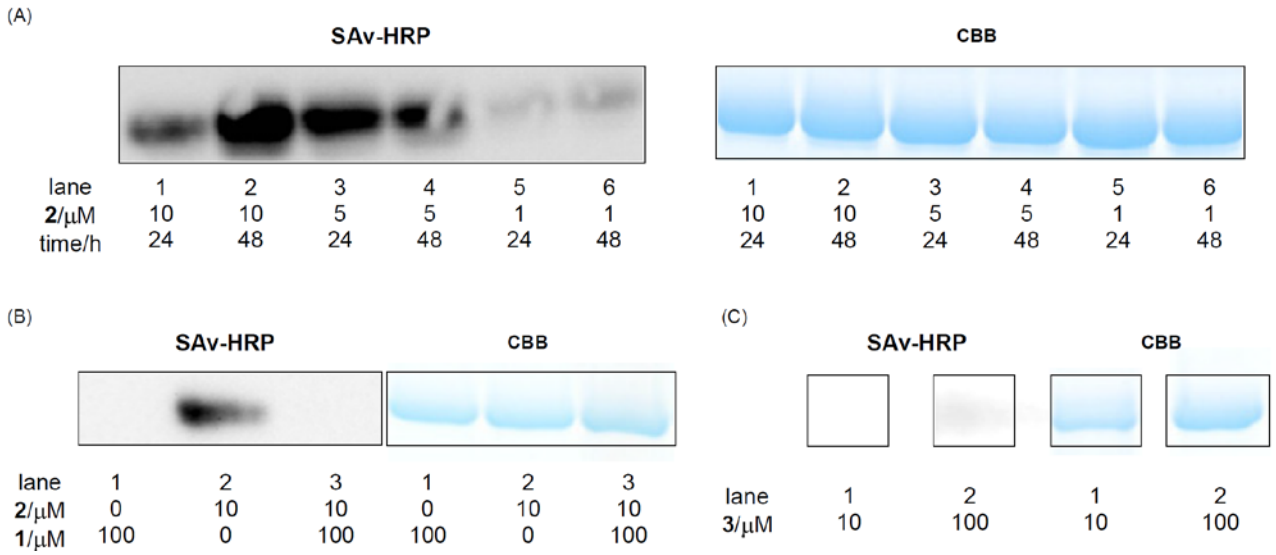


Fig. 4 SDS-PAGE analyses for the labeling of hDAO with the SEAL reagent. (A) hDAO (10 μM) with stabilizers (1 μM FAD, 10 μM dithiothreitol, 20 μM sodium benzoate, 5 mM KCl) was incubated with SEAL reagent **2** (10, 5 or 1 μM) in Tris buffer (50 mM, pH 8.3) at 37 °C for 24 or 48 h. (B) hDAO (10 μM) with stabilizers (1 μM FAD, 10 μM dithiothreitol, 20 μM sodium benzoate, 5 mM KCl) was incubated with SEAL reagent **2** and/or inhibitor **1** in Tris buffer (50 mM, pH 8.3) at 37 °C for 48 h. (C) hDAO (10 μM) with stabilizers (1 μM FAD, 10 μM dithiothreitol, 20 μM sodium benzoate, 5 mM KCl) was incubated with negative control **3** (10 or 100 μM) at 37 °C for 48 h. An original picture of Fig. 4C before band separation is shown in Fig. S1 in ESI.

Graphical Abstract