Supporting Information

Pinpoint Chemical Modification of Asp160 in the 49 kDa Subunit of Bovine Mitochondrial Complex I via a Combination of Ligand-Directed Tosyl Chemistry and Click Chemistry

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Synthesis of AL2	p. 1
Synthesis of biotin–SS–alkyne	p. 4
Synthesis of biotin–SS–ADIBO	p. 5
Figure S1	p. 6
Figure S2	p. 7
Figure S3	p. 8
Figure S4	p. 9
Figure S5	p. 10
Figure S6	p. 11
Figure S7	p. 12
Figure S8	p. 13

Synthesis of AL2

General procedures

^{1}H NMR spectra were recorded at 500 or 400 MHz with Bruker AVANCE III 500 or AVANCE III 400 spectrometers, respectively, using tetramethylsilane (TMS) as the internal standard. ^{13}C NMR spectra were recorded at 125 or 100 MHz. Chemical shift (δ) are given in ppm relative to TMS with coupling constants (*J*) in Hz. The mass spectra were recorded on a Shimadzu LCMS-8040 with ESI source at positive mode. Thin-layer chromatography (TLC) was performed on Merck TLC Plate Silica-gel 60 F^{254}, and the spot was detected by iodine, anis, phosphomolybdic acid, or UV absorbance. Dry solvents were either used as purchased or freshly distilled using common practices where appropriate.

Abbreviations

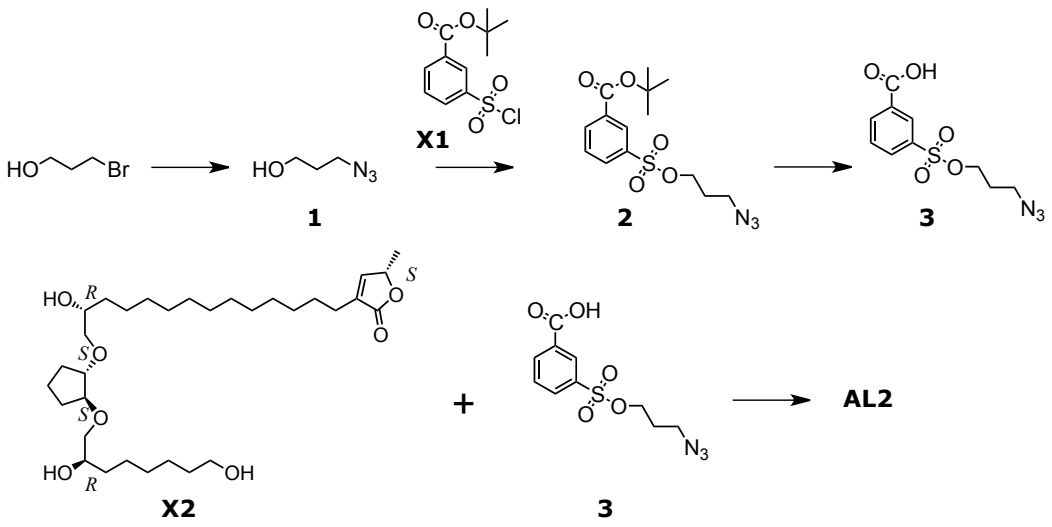
DIPEA, *N,N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; TFA, trifluoroacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

*Outline of the synthesis of **AL2***

The synthetic procedure of acetogenin ligand **AL2** is outlined in Scheme 1. The key intermediates **X1** and **X2** were synthesized as described previously (ref. *11*). 3-Bromo-1-propanol was treated with sodium azide to provide **1**. Reaction of alkoxide of **1** with sulfonyl chloride **X1** and the subsequent deprotection of *tert*-butyl alcohol gave compound **3**. Synthesis of **AL2** was accomplished by esterification of **X2** with **3**.

*Compound **1***

To a solution of 3-bromo-1-propanol (1.00 g, 7.20 mmol) in water, NaN_3 (935 mg, 14.4 mmol) was added at room temperature. After stirring for 22 h at 50ºC, the reaction mixture was cooled to room temperature, extracted with CH_2Cl_2, and dried over anhydrous $MgSO_4$. The crude product was purified by silica gel column chromatography (Wako gel® C-200, 20% EtOAc/hexane) to provide **1** as a colorless oil (708 mg, 7.00 mmol, 97%): ^{1}H NMR (400 MHz; $CDCl_3$): δ 3.75 (t, *J* = 6.0 Hz, 2H), 3.45 (t, *J* = 6.6 Hz, 2H), 2.06 (br s, 1H), 1.84 (tt, *J* = 6.3, 6.3 Hz, 2H).



Scheme 1. *Reagents and conditions*: (a) NaN_3, H_2O, 50ºC, 22 h, 97%; (b) **X1**, DIPEA, DMAP, CH_2Cl_2, rt, 20 h, 25%; (c) TFA, rt, 1 h, 98%; (d) EDC, DMAP, CH_2Cl_2, 0 ºC, 8 h, 70%.

*Compound **2***

To a solution of **1** (202 mg, 2.0 mmol) in anhydrous CH_2Cl_2 (0.5 mL), DIPEA (530 µL, 4.0 mmol) and DMAP (24 mg, 0.200 mmol) were added under N_2 atmosphere at 0°C, and the mixture was stirred for 5 min at 0˚C. Then, **X1** (664 mg, 2.4 mmol) in anhydrous CH_2Cl_2 (4 mL) was added dropwise to the mixture and stirred for 10 min at 0˚C. The reaction mixture was allowed to stir for 20 h at room temperature. The organic solvents were evaporated, and the crude residue was purified by silica gel column chromatography (Wako gel® C-200, 5% to 10% EtOAc/hexane) to provide **2** as a colorless oil (170 mg, 0.498mmol, 25%): ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.50 (dd, *J* = 1.7, 1.7 Hz, 1H), 8.28 (ddd, *J* = 7.8, 1.3, 1.3 Hz, 1H), 8.07 (ddd, *J* = 7.9, 1., 1.3 Hz, 1H), 7.65 (dd, *J* = 7.8, 7.8 Hz, 1H), 4.17 (t, *J* = 6.0 Hz, 2H), 3.41 (t, *J* = 6.4 Hz, 2H), 1.93 (tt, *J* = 6.2, 6.2 Hz, 2H), 1.62 (s, 9H); ^{13}C NMR (100 MHz; $CDCl_3$): δ 163.70, 136.30, 134.66, 133.57, 131.29, 129.44, 128.75, 82.57, 67.51, 47.23, 28.48, 28.13 (3C).

*Compound **3***

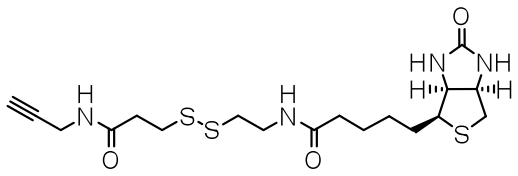
To a solution of **2** (154 mg, 0.451 mmol) in anhydrous CH_2Cl_2 (6 mL), TFA (2 mL) was added. After stirring for 1 h at room temperature, the mixture was co-evaporated with toluene (6 mL) to give compound **3** as a pale yellow solid (126 mg, 0.442 mmol, 98%): ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.66 (dd, *J* = 1.6, 1.6

Hz, 1H), 8.41 (ddd, *J* = 7.8, 1.4, 1.4 Hz, 1H), 8.17 (ddd, *J* = 7.9, 1.5, 1.5 Hz, 1H), 7.74 (dd, *J* = 7.8, 7.8 Hz, 1H), 4.21 (t, *J* = 5.9 Hz, 2H), 3.42 (t, *J* = 6.4 Hz, 2H), 1.95 (tt, *J* = 6.2, 6.2 Hz, 2H); ^{13}C NMR (100 MHz; $CDCl_3$): δ 169.84, 137.06, 135.48, 132.83, 131.00, 129.79, 67.89, 47.40, 28.68.

AL2

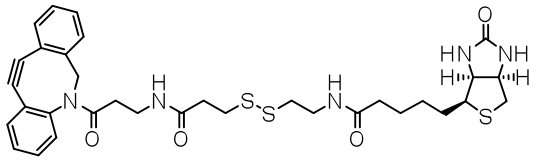
To a solution of **X2** (18 mg, 0.032 mmol) and **3** (11 mg, 0.039 mmol) in anhydrous CH_2Cl_2 (1 mL), EDC (12 mg, 0.065 mmol) and DMAP (4 mg, 0.032 mmol) were added at 0°C under N_2 atmosphere. After stirring for 8 h at 0°C, the reaction was quenched with saturated aqueous NH_4Cl, extracted with CH_2Cl_2, washed with brine and dried over anhydrous $MgSO_4$. The crude product was purified by silica gel column chromatography (Wako gel® C-200, 40% to 60% EtOAc/hexane) to provide **AL2** as a pale yellow oil (10 mg, 0.012 mmol, 70%): ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.56 (dd, *J* = 1.6, 1.6 Hz, 1H), 8.33 (ddd, *J* = 7.9, 1.3, 1.3 Hz, 1H), 8.10 (ddd, *J* = 4.0, 1.4, 1.4 Hz, 1H), 7.68 (dd, *J* = 7.9, 7.9 Hz, 1H), 6.98 (m, 1H), 4.99 (dq, *J* = 6.8, 1.7 Hz, 1H), 4.36 (t, *J* = 6.7 Hz, 1H), 4.19 (t, *J* = 5.9 Hz, 1H), 3.80-3.78 (m, *J* = 2H), 3.75-3.72 (m, 2H), 3.53 (dd, *J* = 9.6, 2.9 Hz, 2H), 3.41 (t, *J* = 6.4 Hz, 2H), 3.28 (dd, *J* = 8.1, 3.1 Hz, 1H), 3.25 (dd, *J* = 8.2, 3.1 Hz, 1H), 2.26 (t, *J* = 7.3 Hz, 2H), 1.96-1.88 (m, 4H), 1.81-1.78 (m, 2H), 1.71-1.63 (m, 2H), 1.61-1.52 (m, 4H), 1.41-1.25 (m, 28H), 1.41 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz; $CDCl_3$): δ 174.08, 164.91, 149.04, 136.84, 134.95, 132.29, 131.87, 129.84, 129.11 (2C), 85.92, 85.89, 77.81, 77.59, 74.28, 74.24, 70.91, 70.79, 67.79, 66.19, 47.47, 33.34, 33.20, 29.94, 29.90, 29.82, 29.78, 29.73, 29.53, 29.48, 29.40, 28.80, 28.63, 27.65, 26.12, 25.76, 25.64, 25.41, 21.24, 21.03, 19.44, 14.42; ESI-HRMS (m/z) calcd for $C_{42}H_{67}N_3NaO_{11}S$ $[M+Na]^+$ 844.4394, found 844.4392.

Synthesis of biotin–SS–alkyne



To a solution of propargyl amine (2.18 mg, 36.9 μmol) in MeOH (2 mL), TEA (8.3 μL, 59.4 μmol) and NHS–SS–biotin (10 mg, 19.8 μmol, purchased from Thermo Fisher Scientic) was added, and the mixture was stirred for 24 h at room temperature. Then the mixture was evaporated and purified by silica gel column chromatography (Wako gel® C-200, 5% to 10% MeOH/$CHCl_3$) to give biotin–SS–alkyne as a white powder (4.0 mg, 9.0 μmol, 45%): ^{1}H NMR (500 MHz; MeOD): δ 4.50-4.47 (m, 1H), 4.31-4.28 (m, 1H), 3.95 (d, J = 2.5, 2H), 3.47 (t, J = 6.4, 2H), 3.22-3.18 (m, 1H), 2.95-2.93 (m, 3H), 2.82 (t, J = 6.6, 2H), 2.69 (d, J = 12.7, 1H), 2.62-2.57 (m, 3H), 2.21 (t, J = 7.2, 2H), 1.76-1.55 (m, 4H), 1.47-1.40 (m, 2H); ^{13}C NMR (125 MHz; MeOD): δ 174.82, 171.94, 164.70, 79.12, 70.79, 61.97, 60.24, 55.56, 39.63, 38.12, 37.24, 35.33, 35.04, 33.61, 28.31, 28.09, 28.06, 25.38; ESI-MS (*m/z*) 445.0 ($[M+H]^+$), 467.0 ($[M+Na]^+$).

Synthesis of biotin–SS–ADIBO



To a solution of *N*-(3-aminopropionyl)-5,6-dihydro-11,12-didehydrodibenzo[*b*,*f*]azocine (5.0 mg, 18 μmol) in MeOH (1 mL), TEA (3.8 μL, 27 μmol) and NHS–SS–biotin (4.6 mg, 9.1 μmol, purchased from Thermo Fisher Scientic) was added, and the mixture was stirred for 18 h at room temperature. Then the mixture was evaporated and purified by silica gel column chromatography (Wako gel® C-200, 10% MeOH/$CHCl_3$) to give biotin–SS–ADIBO as a white powder (2.6 mg, 3.9 μmol, 43%): ^{1}H NMR (500 MHz; MeOD): δ 8.14 (br, 1H), 7.79 (br, 1H), 7.67 (d, *J* = 7.4, 1H), 7.50-7.44 (m, 6H), 7.38 (ddd, *J* = 7.4, 7.4, 1.6, 1H), 7.35 (ddd, *J* = 7.5, 7.5, 1.3, 1H), 7.28 (dd, *J* = 7.3, 1.3, 1H), 5.15 (d, *J* = 14.1, 1H), 4.49-4.46 (m, 1H), 4.30-4.28 (m, 1H), 3.48-3.44 (m, 2H), 3.33-3.23 (m, 1H), 3.22-3.14 (m, 2H), 2.91 (dd, *J* = 12.5, 5.0, 1H), 2.83-2.78 (m, 4H), 2.69 (d, *J* = 14.9, 1H), 2.52-2.46 (m, 1H), 2.42 (t, *J* = 7.2, 2H), 2.21 (t, *J* = 7.3, 2H), 2.12-2.06 (m, 1H), 1.76-1.55 (m, 4H), 1.47-1.40 (m, 2H); ^{13}C NMR (125 MHz; MeOD): δ 176.33, 175.09, 173.76, 173.38, 152.78, 149.66, 133.60, 130.62, 130.15, 129.85, 129.39, 129.11, 128.32, 126.72, 124.51, 123.88, 115.83, 109.03, 63.53, 61.80, 57.12, 56.78, 41.19, 39.69, 38.85, 36.93, 36.90, 36.85, 36.61, 35.21, 29.88, 29.62, 26.94; ESI-MS (*m/z*) 666.1 ($[M+H]^+$), 688.1 ($[M+Na]^+$).

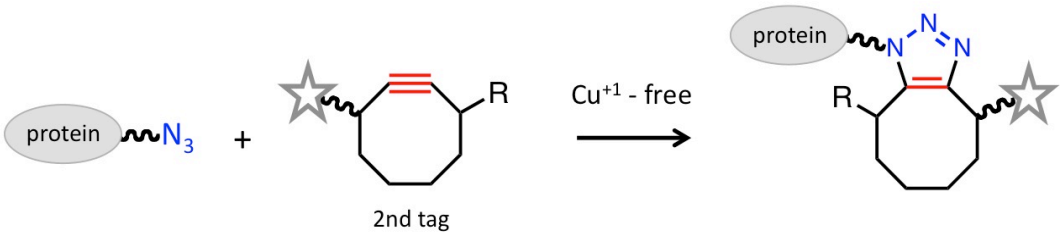


Figure S1

Schematic representation of the Cu^{+1}-free click chemistry between an azido group and a ring-strained cycloalkyne.

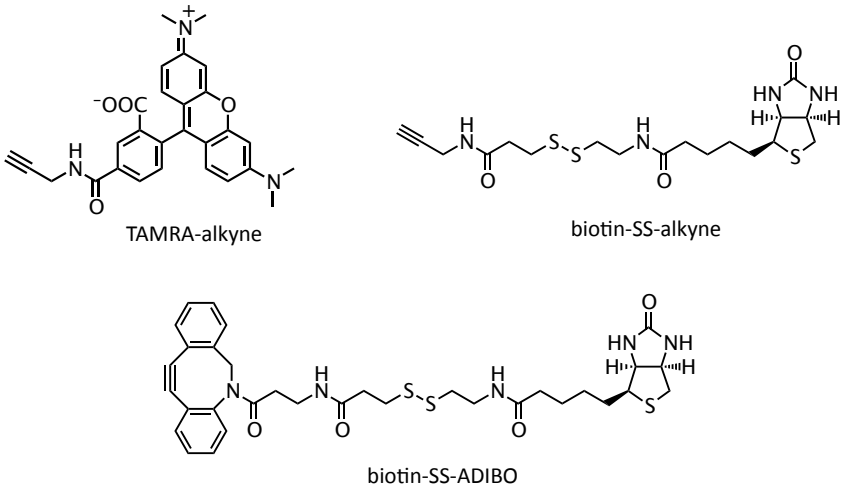


Figure S2

Structures of TAMRA–alkyne, cleavable biotin–SS–alkyne, and biotin–SS–ADIBO.

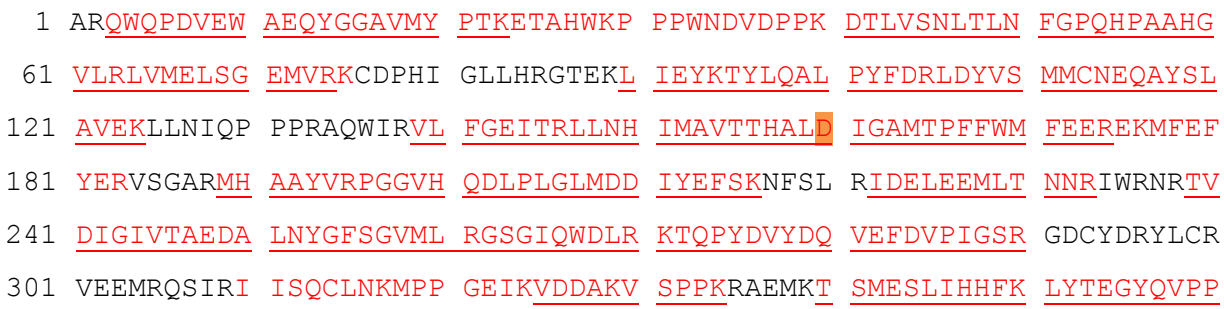


Figure S3

Characterization of the ~50 kDa protein (i.e. the "49-kDa" subunit) of bovine complex I by LC-MS. The sequences of the tryptic digests of the ~50 kDa protein were analyzed by an Orbitrap mass spectrometer, and the identified sequences are shown in *red*. The sequences matched with "high-confidence" in two independent experiments are *underlined*. The azidated residue is highlighted in *orange*. Total 27 peptides were detected and the sequence coverage was 82.3%. The residue number refers to the mature sequence of the bovine 49 kDa subunit (P17694).

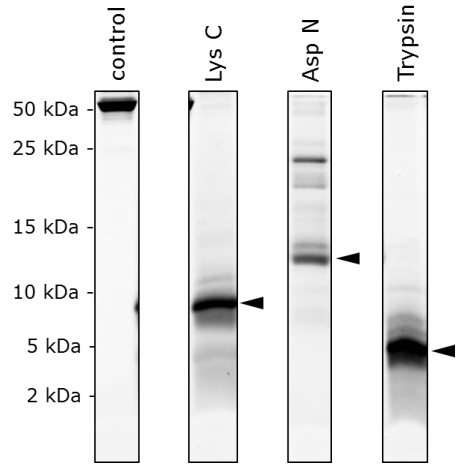


Figure S4

Exhaustive digestion of the azidated 49 kDa subunit. Bovine SMP were azidated via LDT chemistry using 1.0 µM AL2, followed by conjugation with 25 µM TAMRA-alkyne in Click iT reaction buffer kit in the presence of 1% SDS (i.e. via Cu^{+1}–catalized click chemistry). The 49 kDa subunit was partially isolated by SDS-PAGE on a 12.5% Laemmli-type SDS gel and electroelution, and digested with Lys–C, Asp–N, or Trypsin. The digests were analyzed on a Schägger-type SDS gel (16.5% T and 6% C, containing 6.0 M urea).

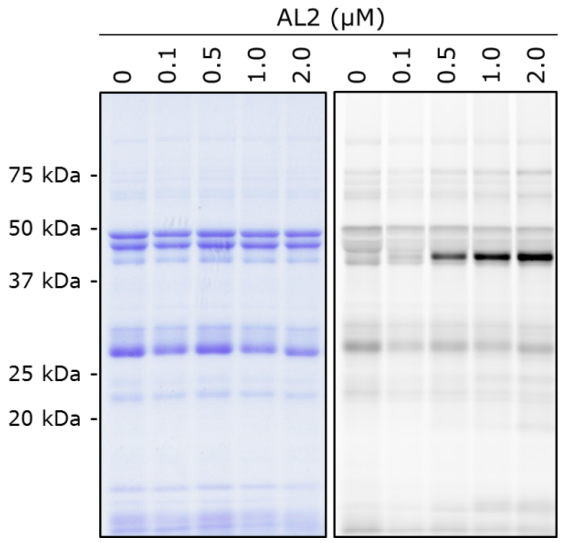


Figure S5

Bovine SMP were azidated via LDT chemistry using different concentrations of AL2 (0~2.0 μM), followed by conjugation with 30 μM TAMRA-alkyne via Cu^{+1}–catalyzed click chemistry in Click iT reaction buffer kit in the presence of 1% SDS at 35˚C for 1 h, and subjected to SDS–PAGE.

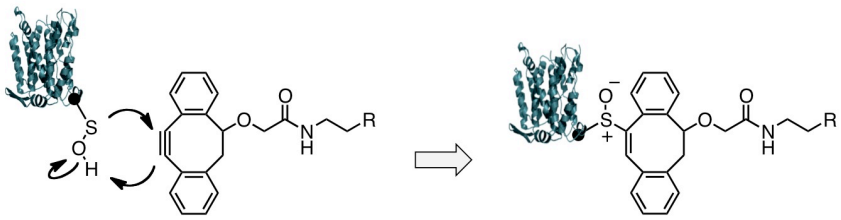


Figure S6

Sulfenic acid (–SOH), which is formed by the reaction of cystein thiol with reactive oxygen species, can readily react to ring-strained cycloalkynes like TAMRA–DIBO (see ref. *28*).

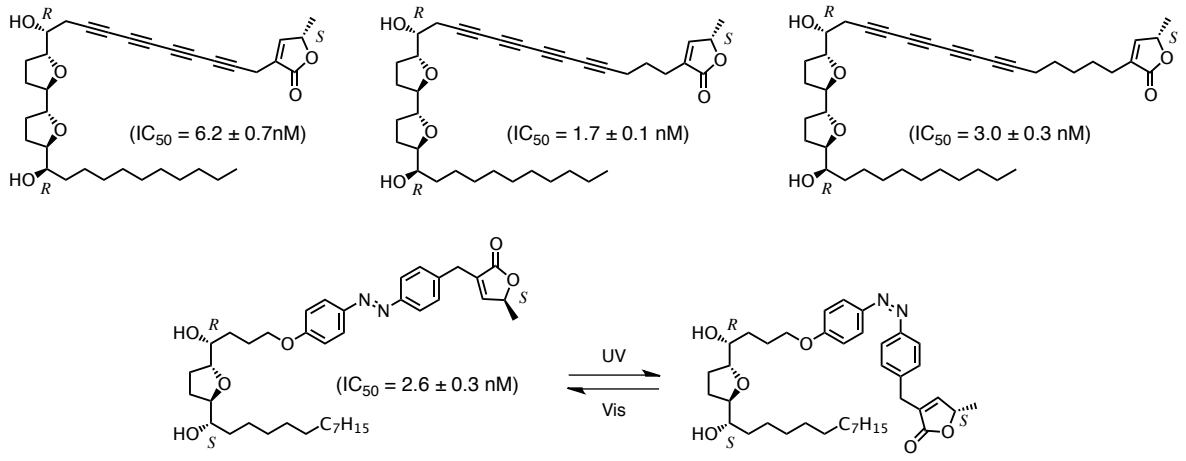


Figure S7

The inhibitory activities of "rigid" acetogenin derivatives, which possess the tetrayne skeleton or the bulky azobenzene unit, are listed (see refs. *30* and *31*). The IC_{50} value of the *cis*-form of azobenzene derivative was unable to be estimated because an actual *trans*:*cis* ratio in an equilibrium state in SMP after UV irradiation could not be determined; nevertheless, it may be nM level (see the above references).

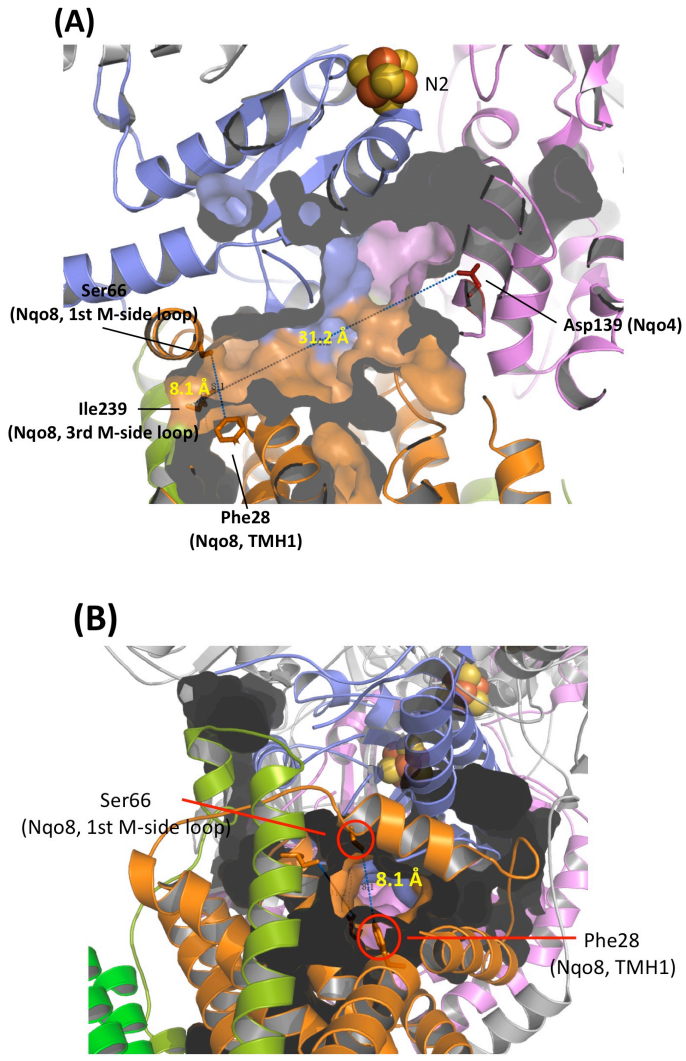


Figure S8

Knowledge about the structure of the quinone/inhibitor binding cavity in *T. thermophilus* complex I (Protein Data Bank entry P17694). (A) Cross-sectional side view of the cavity. The shortest distance between Asp139 in the Nqo4 subunit (Asp160 in bovine 49 kDa subunit) and Ile239 in the Nqo8 was estimated to be 31.2 angstroms. (B) View from the entry point. The distance between Phe28 and Ser66 in the Nqo8, which are located around the entry point of the cavity, was estimated to be 8.1 angstroms. The Nqo4 (49 kDa), Nqo6 (PSST), Nqo7 (ND3), and Nqo8 (ND1) are colored pink, purple, green, and ocher, respectively.