Electronic supplementary information

Derivatization-assisted immunoassays: application for group-specific detection of potent methamphetamine and amphetamine enantiomers

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Preparation of immunogenic hapten–albumin conjugates

Two kinds of immunogenic conjugates, Teoc-(*S*)-MAP–BSA and Teoc-(*S*)-AP–BSA, were prepared in a parallel manner as follows (see Fig. 2 in the text). The starting substances 3-[(2*S*)-2-(methylamino)propyl]phenol [(+)-MAPOH] and 3-[(2*S*)-2-aminopropyl]- phenol [(*S*)-APOH] were obtained as hydrochlorides from Netchem Inc (Brantford, ON, Canada).

Teoc-*O*-succinimidyl (3.0 eq)[1] and 4-(*N*,*N*-dimethylamino)pyridine (0.5 eq) were added to solutions of (*S*)-MAPOH 1 (80 mg) or (*S*)-APOH 1' (72 mg), each dissolved in tetrahydrofuran (THF; 0.5 mL) and pyridine (0.5 mL). These mixtures were stirred at room temperature for 1 h, and then diluted with H_2O and extracted with $CHCl_3$. After removing the solvent, the residues were chromatographed on a silica gel with $CHCl_3$–methanol (30:1) to give Teoc-(*S*)-MAPOH 2 (64 mg) and Teoc-(*S*)-APOH 2' (137 mg), respectively, as a colorless oil. For compound 2, ^{1}H-

NMR (300 MHz; $CDCl_3$), δ: 0.04 (9H, s), 0.87-0.92 (2H, m), 2.01 (3H, s), 2.64-2.72 (2H, m), 2.97 (3H, s), 4.04-4.06 (2H, m), 4.41-4.47 (1H, m), 6.61-6.68 (3H, m), 7.04 (1H, m). HR-MS *m/z*: 308.1690 (Calcd for $C_{16}H_{26}NO_3Si$: 308.1677, $[M-H]^+$); for compound 2', δ: 0.03 (9H, s), 0.85-0.92 (2H, m), 1.00 (3H, d, *J*=6.6 Hz), 2.42-2.72 (2H, m), 3.70-3.81 (1H, m), 3.89-4.05 (2H, m), 6.56-6.60 (3H, m), 7.00 (1H, t, *J*=7.8 Hz). HR-MS *m/z*: 318.1506 (Calcd for $C_{15}H_{24}NO_3SiNa$: 318.1496, $[M+Na]^+$). Glutaric anhydride (20 eq) and 4-(*N*,*N*-dimethylamino)pyridine (0.5 eq) were added to solutions of the carbamate 2 (42 mg) and 2' (80 mg), each dissolved in pyridine (1.0 mL or 2.0 mL), and the mixtures were stirred at room temperature overnight. Then, these reaction mixtures were diluted with 5% HCl (pH 2), and extracted with $CHCl_3$. After removing the solvent, the residues run over a column packed with Supelpak-2 to yield Teoc-(*S*)-MAPOH-suc 3 (26 mg) and Teoc-(*S*)-APOH-suc 3' (56 mg), respectively, as a colorless oil. For compound 3, ^{1}H-NMR (600 MHz; $CDCl_3$), δ: 0.02 (9H, s), 0.70-0.75 (2H, m), 0.97 (3H, s), 1.82 (2H, t, *J*=7.5 Hz), 2.24 (2H, t, *J*=7.5 Hz), 2.44 (2H, t, *J*=7.5 Hz), 2.52-2.54 (2H, m), 3.11 (3H, s), 3.85-3.91 (2H, m), 4.21-4.29 (1H, m), 6.69 (1H, s), 6.71 (1H, s), 6.78-6.88 (1H, m), 7.07 (1H, t, *J*=7.5 Hz). HR-MS *m/z*: 424.2151 (Calcd for $C_{21}H_{34}NO_6Si$: 424.2150, $[M+H]^+$); for compound 3', δ: 0.02 (9H, s), 0.70-0.75 (2H, m), 1.07 (3H, s), 1.92-1.97 (2H, m), 2.38-2.43 (2H, m), 2.59-2.64 (4H, m), 3.78-3.84 (2H, m), 4.01-4.07 (H, m), 6.89 (1H, s), 6.89 (1H, d, *J*=6.0 Hz), 7.04 (1H, d, *J*=6.0 Hz), 7.24 (1H, t, *J*=6.0 Hz). HR-MS *m/z*: 410.2003 (Calcd for $C_{20}H_{32}NO_6Si$: 410.1907, $[M+H]^+$).

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (5.0 eq) and *N*-hydroxysuccinimide (NHS; 5.0 eq) were added to solutions of succinate 3 (25 mg) and 3' (56 mg), each dissolved in 95% 1,4-dioxane (1.0 mL).[2] The mixtures were stirred at room temperature for 2 h, then diluted with water and extracted with ethyl acetate. After removing the solvent, crude NHS esters (4 and 4', respectively) were obtained as a colorless oil, a portion of which [6.3 mg, (12 μmol) and 15 mg (30 μmol), respectively] were then reacted with bovine serum albumin (BSA) [40 mg, (0.60 μmol) or 100 mg, (1.5 μmol), respectively; Sigma–Aldrich (St. Louis, MO, USA)] dissolved in pyridine–PB (1:4, 2.5 mL) with stirring at room temperature for 3 h and then at 4°C overnight. These reaction mixtures were dialyzed as described previously,[3-7] and desired Teoc-(*S*)-MAP–BSA and Teoc-(*S*)-AP–BSA were obtained as 1.7 mg/mL and 3.0 mg/mL solutions in saline. The hapten/BSA coupling molar ratio was determined to be 36:1 and 6:1, respectively, as described above.[3-7]

Preparation of Teoc-(*S*)-MAP–biotin and Teoc-(*S*)-AP–biotin indirect conjugates

As the probe molecules for screening the production of anti-(*S*)-MAP or anti-(*S*)-AP antibodies from hybridomas, we prepared Teoc-(*S*)-MAP–biotin and Teoc-(*S*)-AP–biotin indirect conjugates by labeling Teoc-(*S*)-MAPOH (or -APOH) and biotin moieties together on poly-$_L$-

lysine molecules. This was aimed to avoid possible false-positive bindings by anti-BSA antibodies when we screen hybridoma supernatants with ELISAs using microplates coated with Teoc-(*S*)-MAP (or -AP)–BSA conjugates. The mixtures of Crude NHS ester **4** and **4'** (5.3 mg, 10 μmol) dissolved in 1,4-dioxane (50 μL) and EZ-link NHS-LC-Biotin (Thermo Fisher Scientific; Waltham, MA, USA) (4.5 mg) dissolved in *N*,*N*-dimethylformamide (50 μL) were added to a solution of poly-L-lysine hydrobromide (M_r 4,000–15,000; Sigma–Aldrich) (5.0 mg) in PB (3.0 mL). Subsequently, the mixtures were stirred at room temperature for 1 h and then overnight at 4°C. The resulting solutions were dialyzed against 50 mM PB for 1 day, and adjusted to contain *ca.* 1 mg/mL (calculated as unmodified poly-L-lysine) of the indirect Teoc-(*S*)-MAP–biotin or Teoc-(*S*)-AP–biotin conjugate in G-PBS.

Scheme of immunization of mice for mAbs generation

Five BALB/c and five A/J mice (female, 8 weeks of age) were repeatedly immunized with Teoc-(*S*)-MAP–BSA and Teoc-(*S*)-AP–BSA as shown in the Fig. S1. Selected mice (shadowed), which showed favorable humoral immune responses, were received a final immunization, and their splenocytes were used for the cell fusion experiments.

ELISA for screening hybridomas secreting desirable antibodies

Costar #3590 96-well microplates (Corning; Corning, NY, USA) were coated with a 5.0-μg/mL solution of streptavidin (Thermo Fisher Scientific) in 0.10 M carbonate buffer (pH 8.6) (100 μL/well) overnight at 4°C, and then blocked with M-PBS at 37°C for 60 min. The wells were washed three times with T-PBS, and then a solution of the Teoc-(*S*)-MAP–biotin or Teoc-(*S*)-AP–biotin indirect conjugate (see above) in PVG-PBS (1 μg mL^{-1}; 100 μL per well) was added and incubated at 37°C for 60 min. After washing similarly, hybridoma supernatants diluted with G-PBS (100 μL per well) were added, mixed, and the plates were incubated at 37°C for further 60 min. Subsequently, the wells were washed and probed with a 160-ng mL^{-1} solution of goat anti-mouse IgG (Fc-specific) antibody conjugated with peroxidase (POD) (Jackson ImmunoResearch; West Grove, PA, USA) diluted in G-PBS (100 μl per well). After incubation at 37°C for 30 min, the wells were washed and the POD activity was determined colorimetrically at 490 nm using *o*-phenylenediamine as the hydrogen donor.[3-7]

Synthesis of Teoc derivatives

(a) Teoc-(S)-MAP

Teoc-*O*-succinimidyl (3.0 eq) was added to a solution of (*S*)-MAP hydrochloride (152 mg) dissolved in THF (0.5 mL) and pyridine (0.5 mL). This mixture was stirred at room temperature for 1 h. The crude product was chromatographed on silica gel with hexane–ethyl acetate (1 : 1)

to yield Teoc-(*S*)-MAP (167 mg) as a white solid. HR-MS *m/z*: 316.1705 (Calcd for $C_{16}H_{27}NO_2NaSi$: 316.1770, $[M+Na]^+$).

(b) Teoc-(S)-AP

(*S*)-AP sulfate (5 mg) dissolved in H_2O was adjusted to pH 9.5 with 5% Na_2CO_3, and then extracted with ethyl acetate. After removing the solvent, the residue was dissolved in THF (0.5 mL) and pyridine (0.5 mL), and Teoc-*O*-succinimidyl (3.0 eq) was added. This mixture was stirred at room temperature for 1 h, and then the crude product was chromatographed on silica gel with chloroform to yield Teoc-(*S*)-AP (4.5 mg) as a white solid. HR-MS *m/z*: 302.1546 (Calcd for $C_{15}H_{25}NO_2NaSi$: 302.1550, $[M+Na]^+$).

(c) Teoc-tyramine

Teoc-*O*-succinimidyl (3.0 eq) was added to a solution of tyramine hydrochloride (100 mg) dissolved in THF (2.0 mL) and pyridine (2.0 mL). This mixture was stirred at room temperature for 1 h. The crude product was chromatographed on silica gel with chloroform–methanol (30 : 1) to yield Teoc-tyramine (87 mg) as a white solid. HR-MS *m/z*: 304.1338 (Calcd for $C_{14}H_{23}NO_3NaSi$: 304.1343, $[M+Na]^+$).

(d) Teoc-dopamine

Teoc-*O*-succinimidyl (3.0 eq) was added to solutions of dopamine hydrochloride (100 mg) dissolved in THF (2.0 mL) and pyridine (2.0 mL). This mixture was stirred at room temperature for 1 h. The crude product was chromatographed on silica gel with chloroform–methanol (30 : 1) to yield Teoc- dopamine (52 mg) as a white solid. HR-MS *m/z*: 296.1323 (Calcd for $C_{14}H_{22}NO_4Si$: 296.1324, $[M-H]^+$).

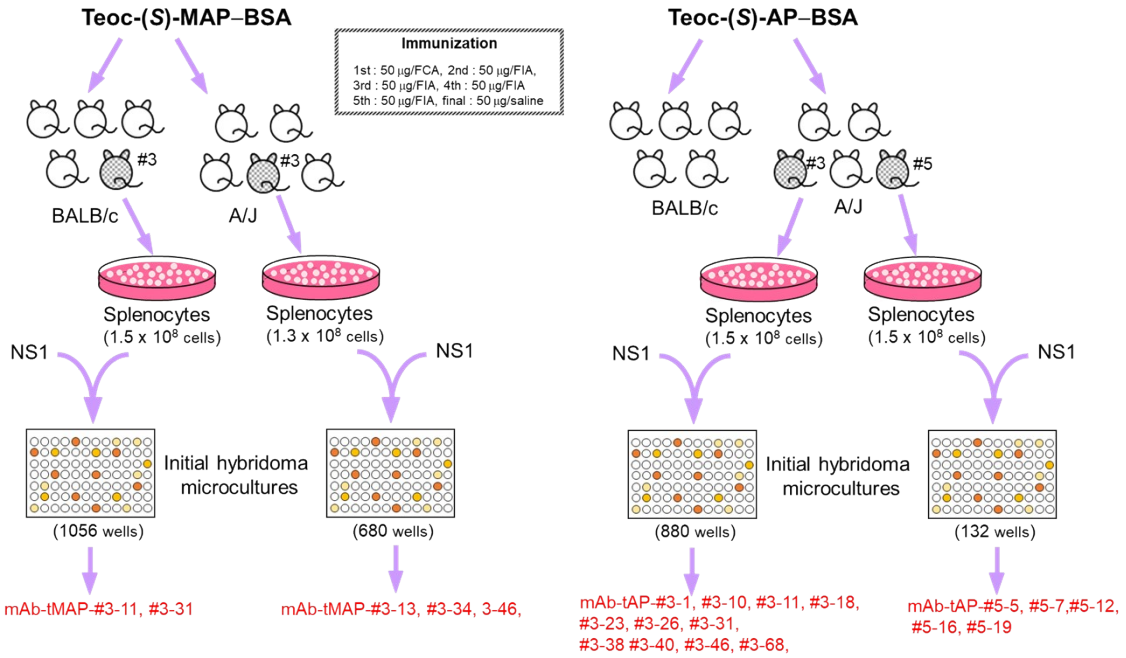

Fig. S1 Schematic illustrations of the immunization of animals, cell fusion, and production of monoclonal antibodies. Each mouse was immunized with subcutaneous injection of 50 μg Teoc-(*S*)-MAP–BSA or Teoc-(*S*)-AP–BSA at multiple sites on the back with an emulsion of Freund's complete adjuvant (FCA). Then, booster immunizations were given 4 times (biweekly interval) with adjuvant (FIA) and saline (1:1, 0.2 mL). The selected four mice (shadowed) that showed favorable humoral responses were received the final immunization with 50 μg Teoc-(*S*)-MAP–BSA or Teoc-(*S*)-AP–BSA in their spleen. The splenocytes were collected from these mice and fused with NS1 myeloma. Number of initial hybridoma microcultures and abbreviations of finally established antibody-secreting hybridoma clones, concerning each fusion experiment, are shown.

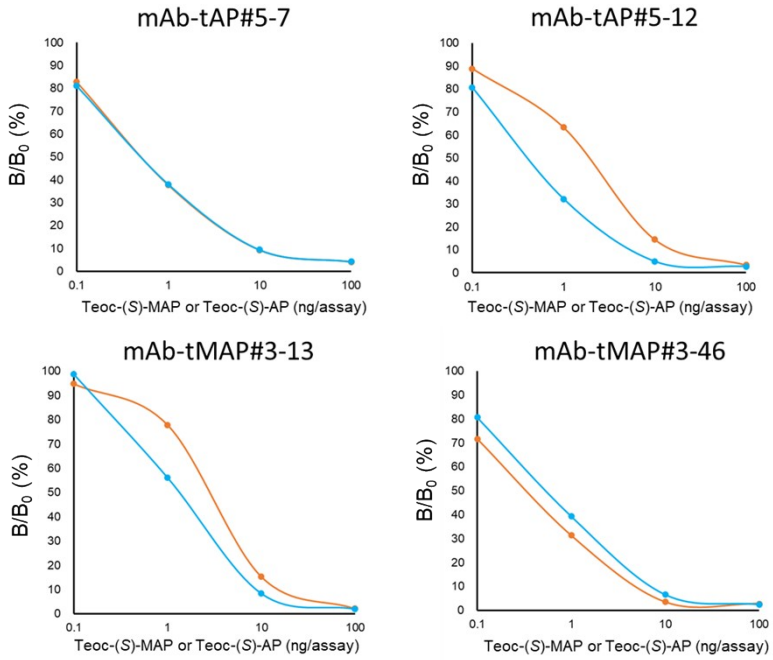

Fig. S2 Dose–response curves of the competitive ELISA for Teoc-(*S*)-MAP (ᔓ) and Teoc-(*S*)-AP (ᔓ) using four selected mAbs.

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