


Evaluation of carboxamide-type synthetic cannabinoids as CB₁/CB₂ receptor agonists: difference between the enantiomers

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Abstract Recently, carboxamide-type synthetic cannabinoids have been distributed globally as new psychoactive substances (NPS). Some of these compounds possess asymmetric carbon, which is derived from an amide moiety composed of amino acid derivatives (i.e., amides or esters of amino acids). In this study, we synthesized both enantiomers of synthetic cannabinoids, *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(2-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA 2-fluorobenzyl isomer), *N*-(1-amino-1-oxo-3-phenylpropan-2-yl)-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide (APP-CHMINACA), ethyl [1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]valinate (5F-EMB-PINACA), ethyl [1-(4-fluorobenzyl)-1*H*-indazole-3-carbonyl]valinate (EMB-FUBINACA), and methyl 2-[1-(4-fluorobenzyl)-1*H*-indole-3-carboxamido]-3,3-dimethylbutanoate (MDMB-FUBICA), which were reported as NPS found in Europe from 2014 to 2015, to evaluate their activities as CB₁/CB₂ receptor agonists. With the exception of (*R*) MDMB-FUBICA, all of the tested enantiomers were assumed to be agonists of both CB₁ and CB₂ receptors, and the EC₅₀ values of the (*S*)-enantiomers for the CB₁ receptors were about five times lower than those of (*R*)-enantiomers. (*R*) MDMB-FUBICA was shown to function as an agonist of the CB₂ receptor, but lacks CB₁ receptor activity. To the best of our knowledge, this is the first report to show that the (*R*)-enantiomers of the carboxamide-type synthetic cannabinoids have the potency to activate CB₁ and CB₂ receptors. The findings presented here shed light on the pharmacological

properties of these carboxamide-type synthetic cannabinoids in forensic cases.

Keywords Carboxamide-type synthetic cannabinoid · Enantiomeric difference in activity · CB₁/CB₂ receptor agonist · AB-FUBINACA 2-fluorobenzyl isomer · APP-CHMINACA · 5F-EMB-PINACA

Introduction

In recent years, several new psychoactive substances (NPS) have been newly identified from illicit drug products, one after another, and have been abused as legal alternatives to scheduled drugs [1]. One of the most popular classes of NPS is synthetic cannabinoids, which are recreationally used as substitutes for *Cannabis sativa*. Forensic cases of synthetic cannabinoid use have increased recently; the adverse effects of indazole-carboxamide-type synthetic cannabinoids, especially, present severe social problems [2].

In a previous study, we developed a method to separate enantiomers of synthetic cannabinoids by liquid chromatography–mass spectrometry (LC–MS) [3]. In the case of NPS classified into cathinone and phenethylamine groups, several reports have concluded that (*S*)-enantiomers are more potent than (*R*)-enantiomers [4–7]. Among the carboxamide-type synthetic cannabinoids, some compounds possess asymmetric carbon, which is derived from an amide moiety composed of amino acid derivatives (i.e., amides or esters of amino acids). These classes of compounds were first developed by Pfizer as potential therapeutic drugs; however, their patent only includes (*S*)-enantiomers, even though the structures of these compounds have chiral centers [8]. Some previous

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studies reported the cannabimimetic activities of the chiral synthetic cannabinoids, but they all investigated only the (*S*)-enantiomers, and thus the pharmaceutical activities of the (*R*)-enantiomers remain unknown [9].

In this study, we synthesized both enantiomers of *N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(2-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA 2-fluorobenzyl isomer), *N*-(1-amino-1-oxo-3-phenylpropan-2-yl)-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide (APP-CHMINACA), ethyl [1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]valinate (5F-EMB-PINACA), ethyl [1-(4-fluorobenzyl)-1*H*-indazole-3-carbonyl]valinate (EMB-FUBINACA), and methyl 2-[1-(4-fluorobenzyl)-1*H*-indole-3-carboxamido]-3,3-dimethylbutanoate (MDMB-FUBICA) to evaluate their potency to activate CB₁ and CB₂ cannabinoid receptors.

Materials and methods

Reagents

Methyl 1*H*-indazole-3-carboxylate and (bromomethyl)cyclohexane were purchased from Sigma-Aldrich (St. Louis, MO, USA); 1-bromo-5-fluoropentane from Fluorochem Ltd. (Hadfield, UK); *D*-valine ethyl ester hydrochloride, *L*-valine ethyl ester hydrochloride, and *D*-valine amide hydrochloride from Combi-Blocks Inc. (San Diego, CA, USA); *D*-phenylalanine amide hydrochloride from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). All other reagents used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded using an ECZS-400 spectrometer (JEOL Resonance, Tokyo, Japan) with dimethyl sulfoxide (DMSO)-*d*₆ as the solvent. The chemical shifts δ were recorded in ppm relative to tetramethylsilane (¹H: $\delta = 0$ ppm, ¹³C: $\delta = 0$ ppm) or the solvent (¹³C: $\delta = 39.5$ ppm) as an internal standard. The compounds were assigned by ¹H NMR, ¹³C NMR, distortionless enhancement by polarization transfer, ¹H-¹³C heteronuclear multiple quantum coherence, ¹H-¹³C heteronuclear multiple-bond correlation, and ¹H-¹H correlation spectroscopy.

Chiral chromatography

Chirality of the compounds was confirmed by LC–MS as described previously [3]. A CHIRALPAK AZ-3R column (3.0 μ m particle size, 150 \times 2.1 mm i.d.) (Daicel Corporation, Osaka, Japan) was used, and the mobile phase was

composed of H₂O/acetonitrile (55:45, v/v) under isocratic conditions. The flow rate of the mobile phase was 0.3 mL/min, and the injection volume was 1 μ L. The column temperature was 40 °C. The stock standard solutions were prepared as described previously [3]. When necessary, the sample solutions and the stock standard solutions were diluted by the mobile phase for the liquid chromatography–high-resolution-mass spectrometry (LC–HR-MS) analysis [3].

Chemical synthesis

The synthetic pathway of these enantiomers is shown in Fig. 1. All of the target compounds were made from methyl 1*H*-indazole-3-carboxylate (compound 1) or methyl 1*H*-indole-3-carboxylate (compound 2). The enantiomers of the target compounds were synthesized by a slightly modified version of a previously described method [3, 10]. Compound 1 or 2 was *N*-alkylated by alkyl halide under basic conditions and yielded compounds that were deprotected by hydrolysis of the methyl group. The target compounds were synthesized by chlorination of the carboxylic acids by oxalyl chloride and then amidation with the amino acid derivatives. The chemical structures of the compounds dealt with in this study are shown in Fig. 2.

N-Alkylation of methyl 1*H*-indazole/indole-3-carboxylate

Compound 5 was synthesized as described previously [3]. Compounds 3, 4, 6, and 7 were prepared in the same manner, except for the starting compounds. To a solution of compound 1 or 2 in tetrahydrofuran (THF), potassium *tert*-butoxide (*t*-BuOK) (1.2 equivalent) was added. To the flask containing the solution, alkyl halide (1.5 equivalent) was added and stirred at room temperature for more than 12 h. Ethyl acetate and distilled water were added to the solution, and the organic layer was separated from the aqueous layer. The organic layer was then washed with saturated aqueous NaCl and dried with anhydrous MgSO₄. The solution was filtered, and the solvent was removed under a vacuum. The residue was purified by an Isolela Speckt System (Biotage, Tokyo, Japan) on a SNAP Ultra column (Biotage) with ethyl acetate in *n*-hexane as the mobile phase. The solvent of the obtained fraction was removed under a vacuum to yield compounds 3, 4, 5, 6 or 7.

Hydrolysis of methyl *N*-alkyl-1*H*-indazole-3-carboxylate

Synthesis of compound 10 was performed as described previously [3]. Compounds 8, 9, and 11 were synthesized similarly, except for the starting compounds. To a flask containing compounds 3, 4, or 6, THF, ethanol, and 1 M NaOH_(aq) (approximately 2.5 mL/1 mmol of the material compounds)

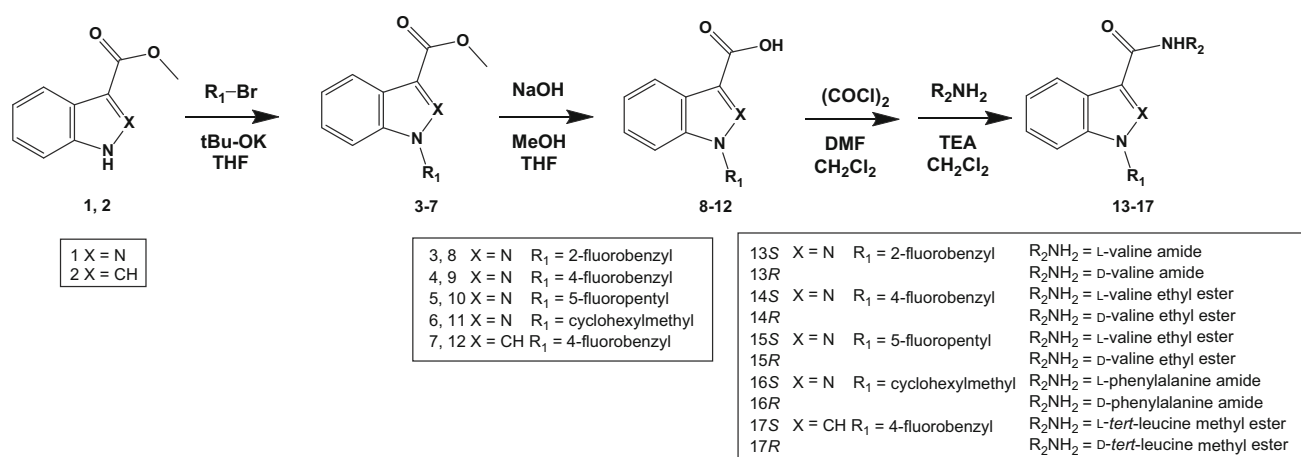


Fig. 1 Procedures for synthesizing the enantiomers dealt with in this study. *tBu-OK* potassium *tert*-butoxide, *THF* tetrahydrofuran, *DMF* *N,N*-dimethylformamide, $(COCl)_2$ oxalyl chloride, *TEA* triethylamine, *MeOH* methanol

were added and stirred at room temperature for more than 16 h. After the reaction, the organic solvents were removed under a vacuum. Ethyl acetate and 20% Na_2CO_3 in H_2O were added to the solution and stirred, and the solution was allowed to separate. The organic layer was extracted by 20% Na_2CO_3 in H_2O again, and the aqueous layers were combined. The organic layer was extracted twice with 1 M NaOH in H_2O . The aqueous layers were combined and neutralized with concentrated HCl, extracted with ethyl acetate twice, and then the organic layer was washed twice with brine. Anhydrous $MgSO_4$ was added to the organic layer and removed under a vacuum to yield compounds **8**, **9**, or **11**.

Hydrolysis of methyl *N*-alkyl-1*H*-indole-3-carboxylate

Compound **12** was synthesized by the hydrolysis of compound **7**. To a flask containing compound **7**, THF, ethanol, methanol, and 10 M $NaOH_{(aq)}$ (approximately 2 mL/1 mmol of compound **7**) were added and stirred at room temperature for 2.5 h. After the reaction, the organic solvents were removed under a vacuum. Ethyl acetate was added to the solution and mixed well, and the solution was allowed to separate. The organic layer was extracted twice with 1 M $NaOH_{(aq)}$, and the aqueous layers were then combined. The aqueous layer was neutralized with HCl and extracted with ethyl acetate twice. The organic layer was then washed twice with brine. Subsequently, anhydrous $MgSO_4$ was added to the combined organic layer and removed under a vacuum to yield compound **12**.

(*S*)-*N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(2-fluorobenzyl)-1*H*-indazole-3-carboxamide (**13S**)

To a flask containing compound **8** in dichloromethane (20 mL/1 mmol of compound **8**), *N,N*-dimethylformamide

(20 μ L/1 mmol) and oxalyl chloride (2.5 equivalent) were added and stirred at room temperature for 30 min. The solvent was then removed under a vacuum to obtain 1-(2-fluorobenzyl)-1*H*-indazole-3-carbonyl chloride as a hazy oily residue. The residue was dissolved in dichloromethane (10 mL), and L-valine amide hydrochloride (1.1 equivalent) was added. Triethylamine (1 mmol) was added to the mixture and then the resulting solution was stirred at room temperature for 90 min. The solvent was removed under reduced pressure and dissolved in ethyl acetate. The solvent was separated with water and washed with 0.1 M HCl, saturated $NaHCO_3$, and saturated NaCl. Drying with $MgSO_4$ and removal of the solvent afforded compound **13S** as a fibrous white solid.

1H NMR ($DMSO-d_6$): δ 8.19 (1H, dd, $J = 8.0, 1.0$ Hz), 7.77 (1H, d, $J = 8.5$ Hz), 7.69 (1H, d, $J = 9.0$ Hz), 7.65 (1H, s), 7.48 (1H, t-like), 7.43–7.34 (1H, m), 7.30 (1H, dd, $J = 8.0, 7.0$ Hz), 7.26–7.18 (2H, m), 7.17–7.09 (2H, m), 5.84 (2H, s), 4.42 (1H, dd, $J = 9.0, 6.0$ Hz), 2.09 (1H, m), 0.94 (3H, d, $J = 7.0$ Hz), 0.89 (3H, d, $J = 7.0$ Hz). ^{13}C NMR ($DMSO-d_6$): δ 172.5, 161.1, 159.9 (d, $J = 246$ Hz), 140.8, 137.2, 130.1 (d, $J = 8$ Hz), 129.7 (d, $J = 4$ Hz), 127.0, 124.7 (d, $J = 4$ Hz), 123.5 (d, $J = 14$ Hz), 122.7, 122.1, 121.8, 115.5 (d, $J = 21$ Hz), 110.4, 56.8, 46.5 (d, $J = 3$ Hz), 31.2, 19.3, 17.9.

(*R*)-*N*-(1-Amino-3-methyl-1-oxobutan-2-yl)-1-(2-fluorobenzyl)-1*H*-indazole-3-carboxamide (**13R**)

Subjecting compound **8** to a procedure similar to that for **13S**, but substituting D-valine amide hydrochloride for L-valine amide, yielded **13R** as a fibrous white solid.

1H NMR ($DMSO-d_6$): δ 8.20 (1H, d, $J = 8.0$ Hz), 7.77 (1H, d, $J = 8.5$ Hz), 7.71 (1H, d, $J = 9.0$ Hz), 7.66 (1H, s), 7.48 (1H, t-like), 7.42–7.34 (1H, m), 7.30 (1H, t-like),

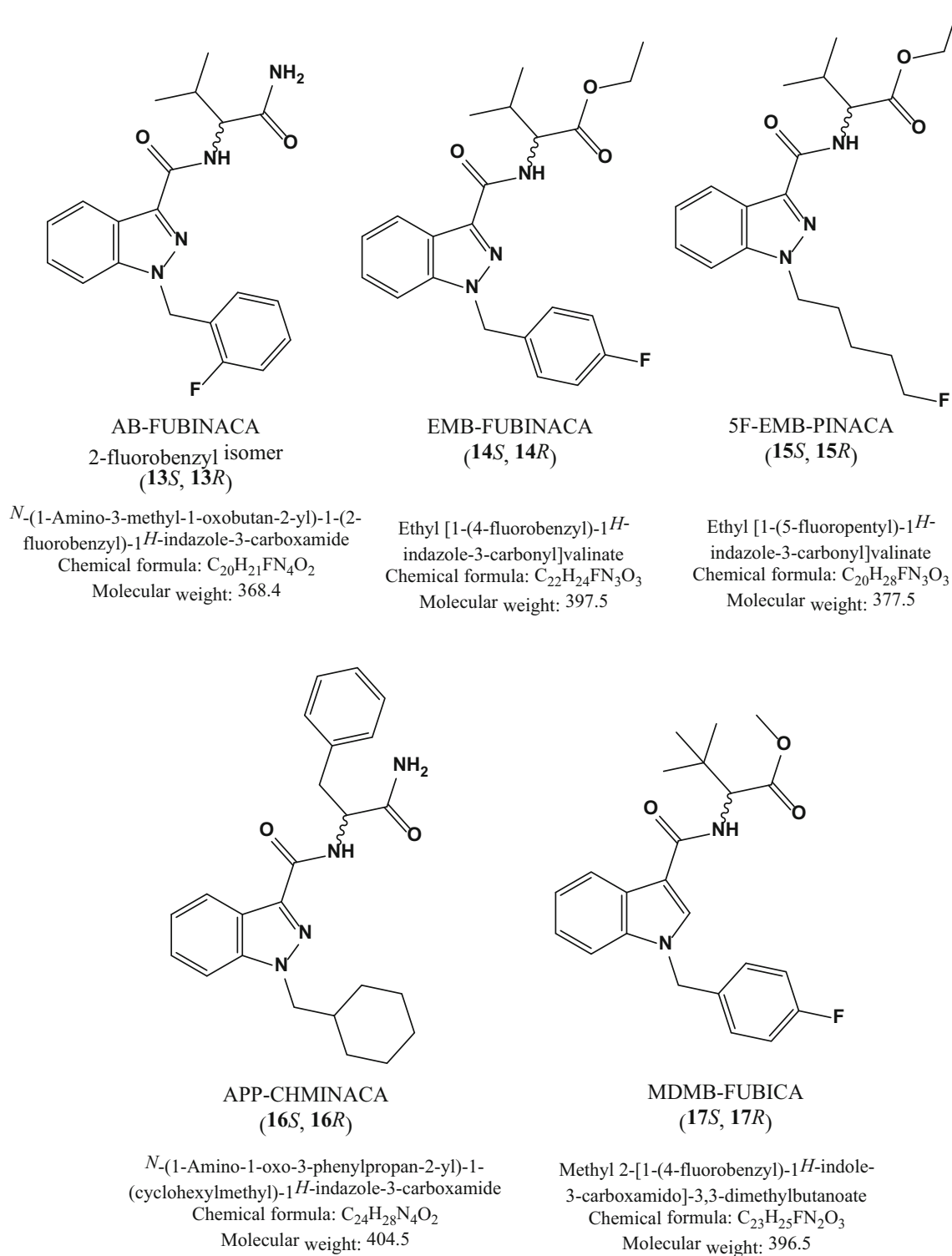


Fig. 2 Chemical structures of the target compounds in this study

7.28–7.20 (2H, m), 7.18–7.10 (2H, m), 5.84 (2H, s), 4.42 (1H, dd, $J = 9.0, 6.0$ Hz), 2.10 (1H, m), 0.94 (3H, d, $J = 7.0$ Hz), 0.89 (3H, d, $J = 7.0$ Hz). ¹³C NMR (DMSO-*d*₆): δ 172.5, 161.2, 159.9 (d, $J = 247$ Hz), 140.8, 137.2,

130.2 (d, $J = 9$ Hz), 129.8 (d, $J = 4$ Hz), 127.0, 124.7 (d, $J = 3$ Hz), 123.6 (d, $J = 15$ Hz), 122.7, 122.1, 121.8, 115.6 (d, $J = 21$ Hz), 110.5, 56.8, 46.5 (d, $J = 3$ Hz), 31.2, 19.4, 18.0.

Ethyl [1-(4-fluorobenzyl)-1H-indazole-3-carbonyl]-L-valinate (14S)

Subjecting compound **9** to a procedure similar to that for **13S**, but substituting L-valine ethyl ester hydrochloride for L-valine amide, yielded **14S** as a clear oily residue.

¹H NMR (DMSO-*d*₆): δ 8.15 (1H, d, *J* = 8.5 Hz), 8.12 (1H, d, *J* = 8.0 Hz), 7.80 (1H, d, *J* = 8.5 Hz), 7.47 (1H, ddd-like), 7.37–7.32 (2H, m), 7.30 (1H, t-like), 7.20–7.14 (2H, m), 5.78 (2H, s), 4.45 (1H, dd, *J* = 8.0, 7.0 Hz), 4.22–4.12 (2H, m), 2.30–2.22 (1H, m), 1.22 (3H, t, *J* = 7.0 Hz), 0.97 (6H, t-like). ¹³C NMR (DMSO-*d*₆): δ 171.5, 161.9, 161.6 (d, *J* = 244 Hz), 140.5, 136.9, 133.0 (d, *J* = 3 Hz), 129.5 (d, *J* = 8 Hz), 127.0, 122.8, 122.4, 121.7, 115.5 (d, *J* = 21 Hz), 110.6, 60.6, 57.4, 51.6, 29.9, 19.0, 18.7, 14.1.

Ethyl [1-(4-fluorobenzyl)-1H-indazole-3-carbonyl]-D-valinate (14R)

Subjecting compound **9** to a procedure similar to that for **13S**, but substituting D-valine ethyl ester hydrochloride for L-valine amide, yielded **14R** as a clear oily residue.

¹H NMR (DMSO-*d*₆): δ 8.15 (1H, d, *J* = 8.5 Hz), 8.12 (1H, d, *J* = 8.5 Hz), 7.80 (1H, d, *J* = 8.5 Hz), 7.46 (1H, ddd-like), 7.37–7.32 (2H, m), 7.29 (1H, t-like), 7.20–7.14 (2H, m), 5.78 (2H, s), 4.45 (1H, dd, *J* = 8.5, 6.5 Hz), 4.22–4.12 (2H, m), 2.30–2.22 (1H, m), 1.22 (3H, t, *J* = 7.0 Hz), 0.97 (6H, t-like). ¹³C NMR (DMSO-*d*₆): δ 171.5, 161.8, 161.6 (d, *J* = 244 Hz), 140.5, 136.9, 133.0 (d, *J* = 3 Hz), 129.5 (d, *J* = 8 Hz), 127.0, 122.8, 122.4, 121.7, 115.5 (d, *J* = 21 Hz), 110.6, 60.6, 57.4, 51.6, 29.9, 19.0, 18.7, 14.1.

Ethyl [1-(5-fluoropentyl)-1H-indazole-3-carbonyl]-L-valinate (15S)

Subjecting compound **10** to a procedure similar to that for **13S**, but substituting L-valine ethyl ester hydrochloride for L-valine amide, yielded **15S** as a clear oily residue.

¹H NMR (DMSO-*d*₆): δ 8.14 (1H, d, *J* = 8.0 Hz), 7.99 (1H, d, *J* = 8.5 Hz), 7.81 (1H, d, *J* = 8.5 Hz), 7.47 (1H, ddd-like), 7.29 (1H, t-like), 4.54 (2H, t, *J* = 7.0 Hz), 4.47 (1H, t, *J* = 6.0 Hz), 4.42 (1H, dd, *J* = 8.5, 6.5 Hz), 4.35 (1H, t, *J* = 6.0 Hz), 4.22–4.12 (2H, m), 2.31–2.21 (1H, m), 1.97–1.88 (2H, m), 1.75–1.61 (2H, m), 1.41–1.31 (2H, m), 1.22 (3H, t, *J* = 7.0 Hz), 0.98 (3H, d, *J* = 4.5 Hz), 0.96 (3H, d, *J* = 4.0 Hz). ¹³C NMR (DMSO-*d*₆): δ 171.5, 161.9, 140.6, 136.2, 126.7, 122.6, 122.1, 121.6, 110.5, 83.6 (d, *J* = 162 Hz), 60.6, 57.2, 48.6, 30.0, 29.3 (d, *J* = 19 Hz), 29.0, 22.0 (d, *J* = 6 Hz), 19.0, 18.6, 14.1.

Ethyl [1-(5-fluoropentyl)-1H-indazole-3-carbonyl]-D-valinate (15R)

Subjecting compound **10** to a procedure similar to that for **13S**, but substituting D-valine ethyl ester hydrochloride for L-valine amide, yielded **15R** as a clear oily residue.

¹H NMR (DMSO-*d*₆): δ 8.14 (1H, d, *J* = 7.5 Hz), 7.99 (1H, d, *J* = 8.0 Hz), 7.81 (1H, d, *J* = 8.5 Hz), 7.47 (1H, ddd-like), 7.29 (1H, t-like), 4.53 (2H, t, *J* = 7.0 Hz), 4.47 (1H, t, *J* = 6.0 Hz), 4.43 (1H, dd, *J* = 8.5, 6.5 Hz), 4.35 (1H, t, *J* = 6.0 Hz), 4.24–4.10 (2H, m), 2.31–2.20 (1H, m), 1.97–1.88 (2H, m), 1.75–1.61 (2H, m), 1.41–1.31 (2H, m), 1.22 (3H, t, *J* = 7.0 Hz), 0.98 (3H, d, *J* = 4.0 Hz), 0.96 (3H, d, *J* = 4.5 Hz). ¹³C NMR (DMSO-*d*₆): δ 171.5, 161.9, 140.6, 136.2, 126.7, 122.6, 122.1, 121.6, 110.5, 83.6 (d, *J* = 162 Hz), 60.6, 57.2, 48.6, 30.0, 29.3 (d, *J* = 19 Hz), 29.0, 22.0 (d, *J* = 6 Hz), 19.0, 18.6, 14.1.

(S)-N-(1-Amino-1-oxo-3-phenylpropan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide (16S)

Subjecting **11** to a procedure similar to that for **13S**, but substituting L-phenylalanine amide hydrochloride for L-valine amide yielded **16S** as a crystalline white solid.

¹H NMR (DMSO-*d*₆): δ 8.11 (1H, d, *J* = 8.0 Hz), 7.90 (1H, d, *J* = 8.0 Hz), 7.76 (1H, d, *J* = 8.5 Hz), 7.67 (1H, s), 7.45–7.41 (1H, m), 7.26–7.14 (7H, m), 4.78–4.72 (1H, m), 4.37–4.26 (2H, m), 3.19–3.05 (2H, m), 1.99–1.87 (1H, m), 1.64–1.60 (3H, m), 1.47 (2H, t-like), 1.23–1.10 (3H, m), 1.07–0.97 (2H, m). ¹³C NMR (DMSO-*d*₆): δ 172.6, 161.3, 141.1, 137.6, 136.4, 129.3, 128.0, 126.5, 126.3, 122.4, 121.8, 121.6, 110.6, 54.5, 53.2, 38.4, 37.7, 30.0, 25.8, 25.2, 25.1.

(R)-N-(1-Amino-1-oxo-3-phenylpropan-2-yl)-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide (16R)

Subjecting compound **11** to a procedure similar to that for **16S**, but substituting D-phenylalanine amide hydrochloride for L-phenylalanine amide hydrochloride, yielded **16R** as a crystalline white solid.

¹H NMR (DMSO-*d*₆): δ 8.11 (1H, d, *J* = 8.0 Hz), 7.90 (1H, d, *J* = 8.0 Hz), 7.76 (1H, d, *J* = 8.5 Hz), 7.66 (1H, s), 7.45–7.41 (1H, m), 7.26–7.14 (7H, m), 4.78–4.72 (1H, m), 4.37–4.26 (2H, m), 3.19–3.05 (2H, m), 1.97–1.88 (1H, m), 1.64–1.60 (3H, m), 1.47 (2H, t-like), 1.24–1.10 (3H, m), 1.07–0.97 (2H, m). ¹³C NMR (DMSO-*d*₆): δ 172.6, 161.3, 141.1, 137.6, 136.4, 129.3, 128.0, 126.5, 126.3, 122.3, 121.8, 121.6, 110.6, 54.5, 53.2, 38.4, 37.7, 30.0, 25.8, 25.2, 25.1.

Methyl (S)-2-[1-(4-fluorobenzyl)-1H-indole-3-carboxamido]-3,3-dimethylbutanoate (17S)

Subjecting **12** to a procedure similar to that for **13S**, but substituting *L-tert-leucine* methyl ester hydrochloride for *L-valine* amide yielded **17S** as a white powder.

¹H NMR (DMSO-*d*₆): δ 8.50 (1H, s), 8.11 (1H, d, *J* = 8.0 Hz), 7.71 (1H, d, *J* = 8.5 Hz), 7.52 (1H, d, *J* = 8.5 Hz), 7.34–7.31 (2H, m), 7.20–7.11 (4H, m, overlapped), 5.47 (2H, s), 4.50 (1H, d, *J* = 8.5 Hz), 3.66 (3H, s), 1.04 (9H, s). ¹³C NMR (DMSO-*d*₆): δ 172.0, 164.3, 161.6 (d, *J* = 244 Hz), 136.0, 133.8 (d, *J* = 3 Hz), 132.1, 129.2 (d, *J* = 8 Hz), 126.9, 122.2, 121.3, 120.9, 115.5 (d, *J* = 22 Hz), 110.6, 109.3, 60.0, 51.4, 48.8, 33.8, 26.8.

Methyl (R)-2-[1-(4-fluorobenzyl)-1H-indole-3-carboxamido]-3,3-dimethylbutanoate (17R)

Subjecting compound **12** to a procedure similar to that for **17S**, but substituting *D-tert-leucine* methyl ester hydrochloride for *L-tert-leucine* methyl ester hydrochloride, yielded **17R** as a white powder.

¹H NMR (DMSO-*d*₆): δ 8.50 (1H, s), 8.11 (1H, d, *J* = 7.0 Hz), 7.71 (1H, d, *J* = 8.5 Hz), 7.53 (1H, d, *J* = 8.0 Hz), 7.34–7.31 (2H, m), 7.20–7.13 (4H, m, overlapped), 5.47 (2H, s), 4.50 (1H, d, *J* = 8.5 Hz), 3.66 (3H, s), 1.04 (9H, s). ¹³C NMR (DMSO-*d*₆): δ 172.0, 164.3, 161.6 (d, *J* = 244 Hz), 136.0, 133.7 (d, *J* = 3 Hz), 132.1, 129.2 (d, *J* = 8 Hz), 126.9, 122.2, 121.2, 120.9, 115.5 (d, *J* = 21 Hz), 110.6, 109.3, 60.0, 51.4, 48.7, 33.8, 26.8.

In vitro assays to evaluate the CB₁/CB₂ receptor activities

For the evaluation of CB₁/CB₂ cannabinoid receptor activity, [³⁵S]GTPγS binding assays were performed. These assays were performed at ADME and Tox. Research Institute, Sekisui Medical Co., Ltd. (Tokai-mura, Ibaraki, Japan). The assay conditions were as described previously [11], except for the tested concentration levels of the compounds ranging from 1 × 10⁻¹¹ to 1 × 10⁻⁴ M. Agonistic activities (EC₅₀ value: concentration showing 50% response) of the test compound to the cannabinoid receptors CB₁ and CB₂ were measured.

Results and discussion

In this study, we synthesized both (*S*)- and (*R*)-enantiomers of synthetic cannabinoids to evaluate their pharmacological properties. All target compounds were reported in an EMCDDA-Europol joint report to be NPS sold and recreationally used in Europe [12, 13]. NMR data for the

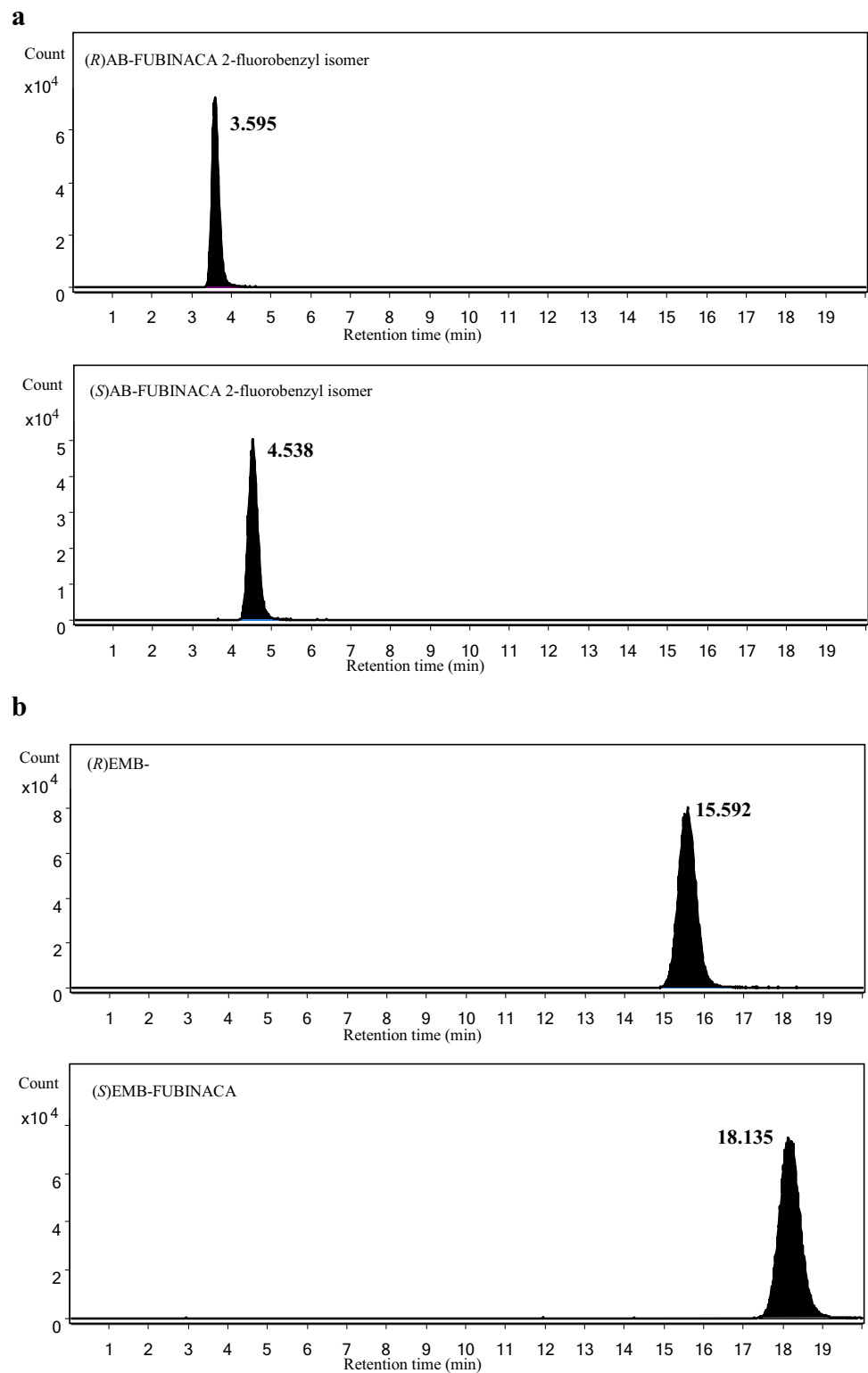
enantiomers were similar, as shown in the section on materials and methods above, and the results are in good agreement with the values expected for their structures.

Figure 3 shows the extracted ion chromatograms for the enantiomers of the target compounds (1 μg/mL solution for both enantiomers) obtained by LC–HR–MS using the method that we developed previously [3]. By the previously developed method, all of the enantiomers of the tested compounds were separated, and no enantio-impurities were found in their chromatograms, except for (*S*) APP-CHMINACA. Approximately less than 2% of (*R*) APP-CHMINACA was confirmed in the extracted ion chromatogram of (*S*) APP-CHMINACA as shown in Fig. 3. However, because (*R*)-enantiomer was much less effective than (*S*)-enantiomer to both CB₁ and CB₂ receptors, it is suspected that the effect of contaminated (*R*)-enantiomer on the EC₅₀ value was almost negligible.

Table 1 shows the results of the [³⁵S]GTPγS binding assays performed in this study. Each compound functioned as both a CB₁ and a CB₂ receptor agonist, except for (*R*) MDMB-FUBICA. The EC₅₀ values of (*S*)-enantiomers for the CB₁ receptors were at least one-fifth of or less than those of (*R*)-enantiomers, which indicates that (*S*)-enantiomers are more potent CB₁ receptor agonists than (*R*)-enantiomers. The (*S*)-enantiomer of APP-CHMINACA could activate CB₁ at a concentration 134 times lower than that for the (*R*)-enantiomer. For MDMB-FUBICA, the CB₁ receptor activity was not confirmed for the (*R*)-enantiomer even at the maximum tested concentration, but the EC₅₀ of the (*S*)-enantiomer was 9.72 × 10⁻⁹ M; the difference in the EC₅₀ levels between the enantiomers of MDMB-FUBICA to activate the CB₁ receptor was more than 10,000-fold, which indicates that the activities for the CB₁ receptor are markedly different depending on the chirality of the compounds. In contrast, we could not observe a clear relevance between EC₅₀ values for the CB₂ receptor and chirality of the compounds. (*R*) APP-CHMINACA and (*R*) MDMB-FUBICA lacked the potential to be CB₁ receptor agonists, but both of their EC₅₀ values for the CB₂ receptor were less than 10⁻⁶ M; all of the target compounds were strong agonists for the CB₂ receptor, irrespective of their chirality.

As for the CB₁/CB₂ selectivity, (*R*)-enantiomers tended to be CB₂-selective and were at least 20-fold more active than for CB₁ for all of the tested compounds. Interestingly, (*R*) MDMB-FUBICA functioned only on the CB₂ receptor as an agonist, while showing no CB₁ activity as mentioned above, although (*S*) MDMB-FUBICA could activate both of these receptors at nanomolar levels. In several preclinical studies, compounds such as JWH-133 and HU308 were used as CB₂-selective ligands [14]. More recently, the synthesis and evaluation of novel CB₂-selective agonists have been repeatedly reported. In these pharmacological or

Fig. 3 Extracted ion chromatograms of **a** AB-FUBINACA 2-fluorobenzyl isomer at m/z 369.1721 ($\Delta = \pm 0.5$ mDa), **b** EMB-FUBINACA at m/z 398.1874 ($\Delta = \pm 0.5$ mDa), **c** 5F-EMB-FUBINACA at m/z 378.2187 ($\Delta = \pm 0.5$ mDa), **d** APP-CHMINACA at m/z 405.2285 ($\Delta = \pm 0.5$ mDa), and **e** MDMB-FUBICA at m/z 397.1922 ($\Delta = \pm 0.5$ mDa), as a function of enantiomers, obtained by liquid chromatography–high-resolution mass spectrometry



medicinal chemistry studies seeking CB₂ agonists, the most selective compound showed a selective index of CB₁/CB₂ of only 100- to 1000-fold. In this study, (*R*) MDMB-FUBICA was shown to have potency to activate the CB₂ receptor signal with an EC₅₀ value of 3.1×10^{-9} M, but

had no [³⁵S]GTPγS binding activity for the CB₁ receptor, even at 1.0×10^{-4} M, which corresponds to a selective index of more than 3.2×10^4 -fold.

The metabolism of MDMB-FUBICA enantiomers has not been reported yet; thus it remains unclear whether

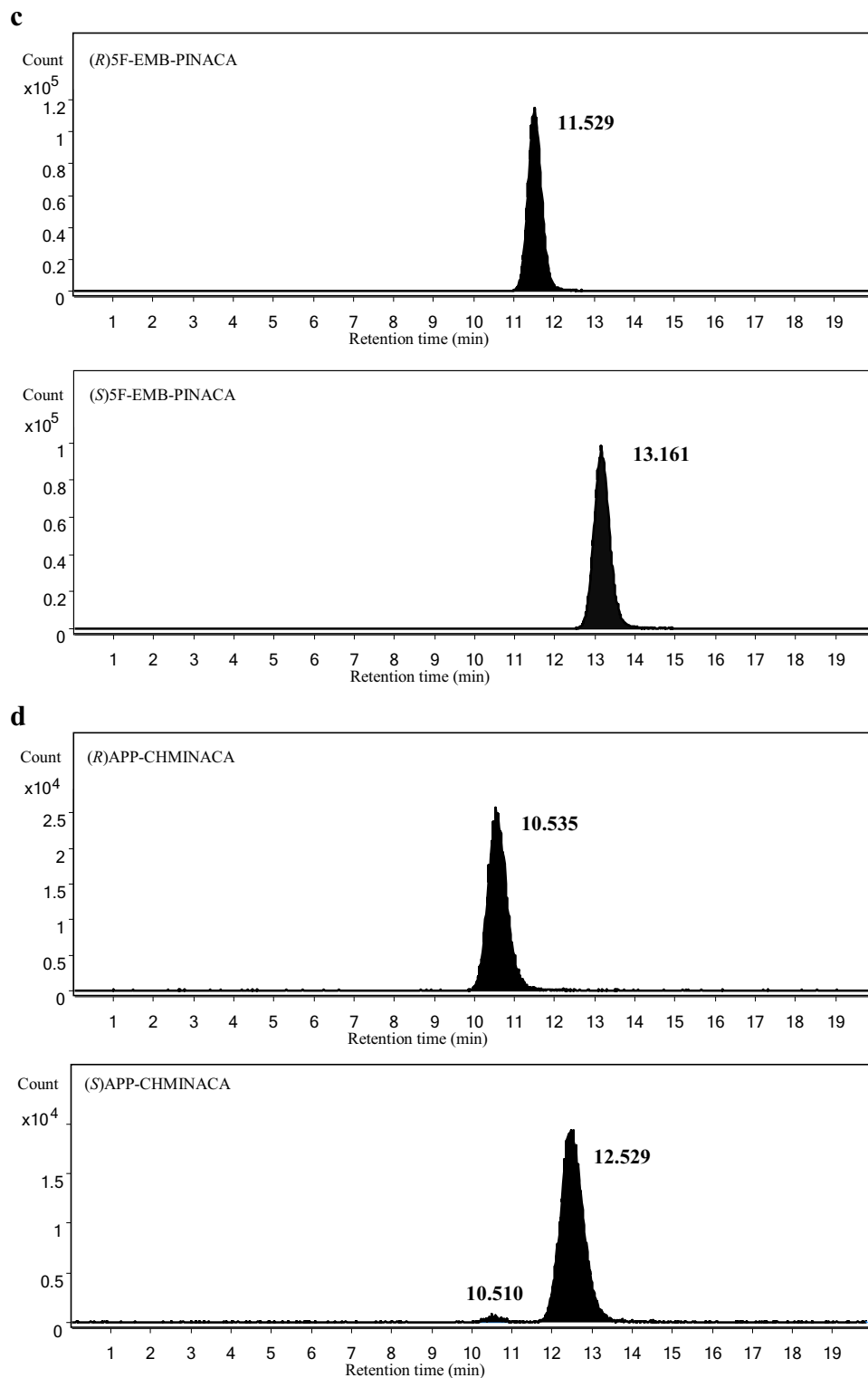
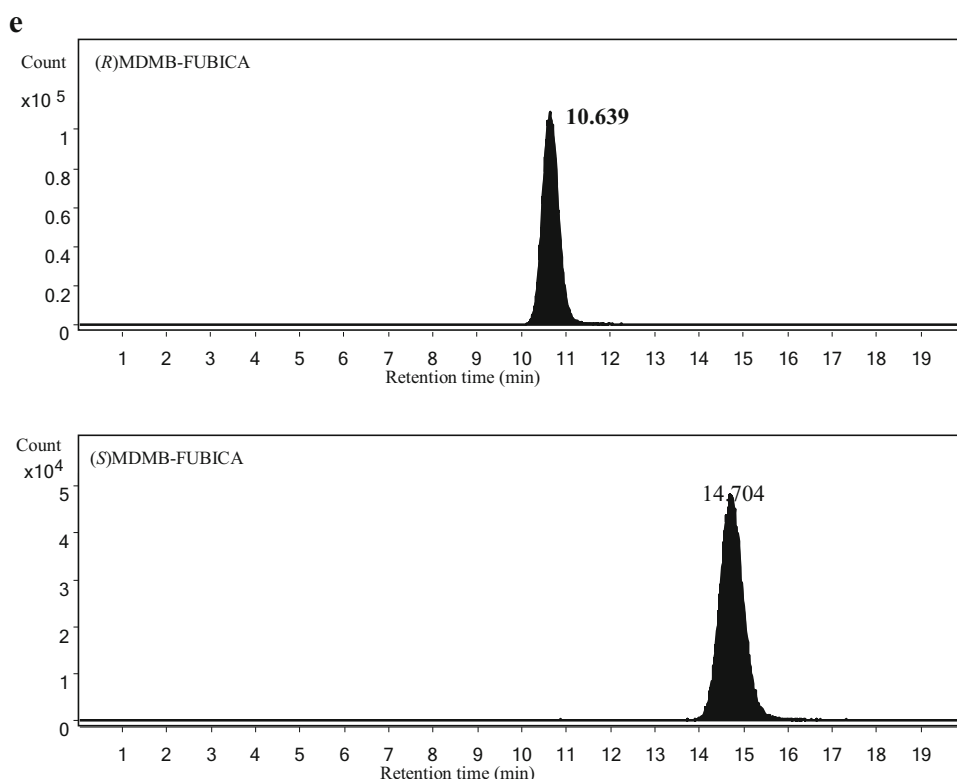


Fig. 3 continued

(*R*) MDMB-FUBICA can mimic the function of the CB₂ receptor agonist when applied to an animal model to evaluate the effect on the central nervous system. Recently,

it was reported that the (*S*)-enantiomer was mainly detected in herbal products containing chiral carboxamide-type synthetic cannabinoids [3, 15]. The cannabimimetic

Fig. 3 continued

**Table 1** Activities of enantiomers of each compound at human CB₁ and CB₂ receptors

Compound name	Human CB ₁ receptor		Human CB ₂ receptor		Selective index ^b (CB ₁ /CB ₂)
	EC ₅₀ (M)	S/R ratio ^a	EC ₅₀ (M)	S/R ratio ^a	
(S)AB-FUBINACA 2-fluorobenzyl isomer	2.92×10^{-9}	0.21	2.44×10^{-9}	3.9	1.20
(R)AB-FUBINACA 2-fluorobenzyl isomer	1.41×10^{-8}		6.27×10^{-10}		22.5
(S)APP-CHMINACA	2.51×10^{-7}	0.0074	8.09×10^{-9}	0.017	31.0
(R)APP-CHMINACA	3.37×10^{-5}		4.90×10^{-7}		68.8
(S)EMB-FUBINACA	4.58×10^{-10}	0.015	2.14×10^{-9}	2.5	0.214
(R)EMB-FUBINACA	3.07×10^{-8}		8.63×10^{-10}		35.6
(S)5F-EMB-PINACA	4.96×10^{-9}	0.14	6.91×10^{-9}	4.1	0.718
(R)5F-EMB-PINACA	3.59×10^{-8}		1.68×10^{-9}		21.4
(S)MDMB-FUBICA	9.72×10^{-9}	<0.0001	1.07×10^{-9}	0.35	9.08
(R)MDMB-FUBICA	$>1.00 \times 10^{-4}$		3.10×10^{-9}		>30,000
CP55940	1.28×10^{-9}		1.54×10^{-10}		8.3

^a S/R ratio is the EC₅₀ value ratio of an (S)-enantiomer to the corresponding (R)-enantiomer

^b Selective index is EC₅₀ ratio of the CB₁ to CB₂

activities of each enantiomer presented in this study may be helpful to precisely recognize forensic cases associated with these synthetic cannabinoids when evaluated with the quantitative data of a specimen analyzed after enantiomeric separation.

Conclusions

The activities of the enantiomers of five synthetic cannabinoids (AB-FUBINACA 2-fluorobenzyl isomer, APP-CHMINACA, 5F-EMB-PINACA, EMB-FUBINACA, and

MDMB-FUBICA) were evaluated by the [³⁵S]GTPγS binding assay. We also confirmed that these enantiomers could be clearly differentiated by chiral-LC–MS. These findings should contribute to better understanding of the forensic cases associated with these synthetic cannabinoids.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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