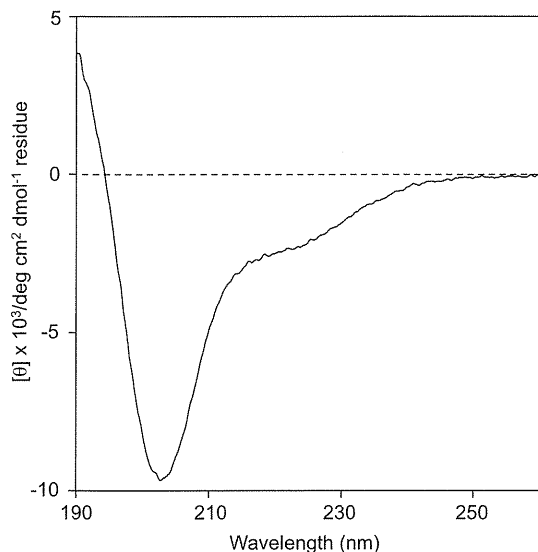

Figure 3. CD spectrum (190–260 nm region) of ***hS*$_{3,7}$*hS*-2^{dOH}** in TFE solution. Peptide concentration: 0.5 mM.

aminoisobutyric acid (Aib) and a cross-linked unit stabilized the helical structures of short Leu-based peptides with structure **A** (Fig. 1)as reported by Grubbs and Verdine.[30] These peptides were able to efficiently catalyze the enantioselective epoxidation of α,β-unsaturated ketones.[31]As peptides **A** contain the LXXLL motif, we considered that any short peptide based on this structure should bind to the VDR and inhibit VDR–coactivator interactions (Fig. 2). Here, we synthesized several stapled heptapeptides containing the LXXLL motif and evaluated their ability to inhibit VDR–coactivator interactions.

The stapled peptides ***S*$_{3,7}$*S*-2**, ***S*$_{3,7}$*R*-2**, ***R*$_{3,7}$*S*-2**, ***R*$_{3,7}$*R*-2**, ***hS*$_{3,7}$*S*-2**, and ***hS*$_{3,7}$*hS*-2** were prepared according to the previously reported methods.[31] Then, their C- and N-termini were deprotected to afford C- and N-terminal-free peptides; that is, **H-*S*$_{3,7}$*S*-2-OH** (**DPI-01**), **H-*S*$_{3,7}$*R*-2-OH** (**DPI-02**), **H-*R*$_{3,7}$*S*-2-OH** (**DPI-03**), **H-*R*$_{3,7}$*R*-2-OH** (**DPI-04**), **H-*hS*$_{3,7}$*S*-2-OH** (**DPI-05**), and **H-*hS*$_{3,7}$*hS*-2-OH** (**DPI-06**), respectively. The stapled peptide ***hS*$_{3,7}$*hS*-2^{dOH}**, which possessed two hydroxyl group side chains, was synthesized from the linear peptide ***hS*$_{3,7}$*hS*-1**[31] via a ruthenium-catalyzed intramolecular ring-closing metathesis reaction followed by osmium catalyzed dihydroxylation.[32] Finally, the C- and N-terminal protecting groups of ***hS*$_{3,7}$*hS*-2^{dOH}** were removed to obtain **H-*hS*$_{3,7}$*hS*-2^{dOH}-OH** (**DPI-07**) (Scheme 1).[33] The isomeric mixture of **DPI-07** was not separated.

The inhibitory activity of the synthetic peptides **DPI-01-07** against VDR-coactivator interactions was evaluated using the EnBio receptor cofactor assay system (RCAS) for the VDR kit (Fujikura Kasei Co., Ltd) according to the manufacturer's instructions.[34] The results are summarized in Table 1. Most of the peptides exhibited weak activity against VDR–coactivator interactions (IC_{50}: 220–610 μM, entries 1–6); however, **DPI-07**, which possesses the side-chain hydroxyl groups, displayed 70-fold greater activity than the other peptides (IC_{50}: 3.2 μM, entry 7).

The dominant conformations of the N- and C-terminal protected peptides ***S*$_{3,7}$*S*-2**, ***S*$_{3,7}$*R*-2**, ***R*$_{3,7}$*S*-2**, ***R*$_{3,7}$*R*-2**, ***hS*$_{3,7}$*S*-2**, ***hS*$_{3,7}$*hS*-2**, and ***hS*$_{3,7}$*hS*-2^{dOH}** were analyzed using the CD spectra in 2,2,2-tri-

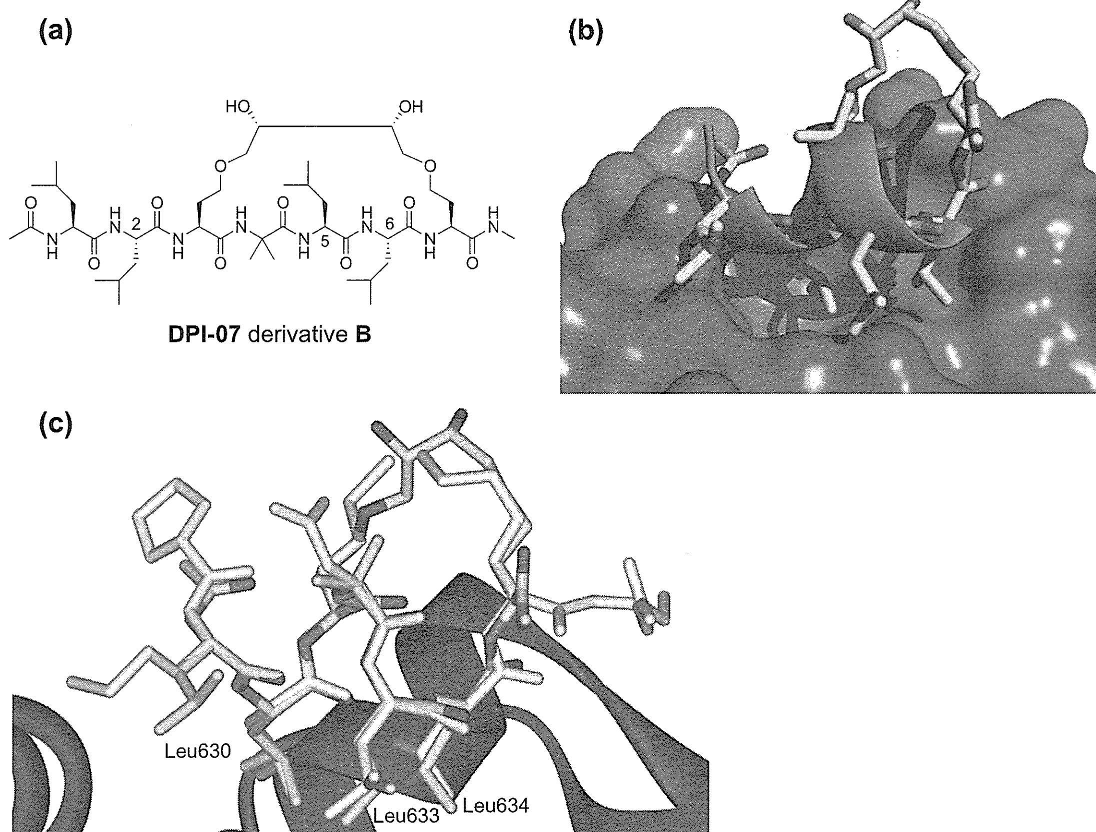

Figure 4. (a) Chemical structure of the modeled **DPI-07** derivative (**B**). (b) Modeled structure of **B** bound to the VDR. The VDR surface is shown in gray. (c) Overlay of the X-ray structure of the coactivator fragment (yellow, PDB ID 3AUN) bound to the VDR and the calculated structure of **B** bound to the VDR.

fluoroethanol (TFE) solution. The CD spectrum of ***hS*$_{3,7}$*hS*-2^{dOH}** showed negative maxima at around 206 and 222 nm, which indicates that ***hS*$_{3,7}$*hS*-2^{dOH}** was folded into a right-handed helical structure as the preferred secondary structure (Fig. 3).[35] The other peptides ***S*$_{3,7}$*S*-2**, ***S*$_{3,7}$*R*-2**, ***R*$_{3,7}$*S*-2**, ***R*$_{3,7}$*R*-2**, ***hS*$_{3,7}$*S*-2**, and ***hS*$_{3,7}$*hS*-2** had the similar CD patterns and intensities to ***hS*$_{3,7}$*hS*-2^{dOH}** in TFE solution, respectively.[30,31,36]

Next, we used the X-ray co-crystal structure (PDB: 3AUN) of VDR bound to the LXXLL motif found in its coactivators to perform docking studies.[37] Accordingly, a docking model of a **DPI-07** derivative (**B**) (Fig. 4a)[38] bound to the VDR was constructed based on a conformational search in *MacroModel* (ver. 9.1). AMBER* was used as the force field, and more than 10,000 conformers for **B** were optimized. The most stable conformation is shown in Figure 4b. In the modeled structure of **B**, the three Leu residues at the 2nd, 5th, and 6th positions are oriented around the hydrophobic region (Ile234, Ile238, Leu259, Ala263, and Val417) of the VDR. The positions of these three Leu residues in **B** displayed good agreement with those of the leucine residues in the LXXLL coactivator motif (Leu630, Leu633, and Leu634) during its binding to the VDR (Fig. 4c).[37] Furthermore, when **B** binds to the VDR its hydroxyl group side chains are located on either side of the Leu-containing motif. These hydroxyl groups could increase the affinity of **DPI-07** for water molecules surrounding the protein, and hence, **DPI-07** strongly binds to the protein. Although all synthetic peptides had the similar helicity, the peptides without any hydroxyl groups did not show strong inhibitory activity (IC_{50}: 220–610 μM). These results also mean that the side-chain hydroxyl groups of the stapled peptide play an important role to develop the strong affinity.

In summary, we developed stapled short helical peptides (**DPI-01-07**) containing an LXXLL motif. The ability of these peptides to inhibit VDR–coactivator interactions was evaluated in an inhibition assay. Peptides **DPI-01-06**, which do not contain hydroxyl groups in their covalent side chains, exhibited weak inhibitory activity (IC_{50}: 220–610 μM), whereas **DPI-07**, which possesses the side-chain hydroxyl groups, displayed strong inhibitory activity (IC_{50}: 3.2 μM). Stapled short helical peptides like **DPI-07** might be useful for treating conditions involving hypersensitivity to $1\alpha,25(OH)_2D_3$. Transcriptional inhibition assays, the derivatization of further peptides, and studies of their effects on other nuclear receptors are currently underway.

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32. In an inert atmosphere, a solution of ***hS*$_{3,7}$*hS*-1** (71 mg, 0.075 mmol) and Grubbs catalyst 2nd generation (32 mg, 0.04 mmol) in CH_2Cl_2 (20 mL) was stirred at room temperature for 12 h. The solution was then poured into water and extracted with CH_2Cl_2. The combined organic layer was dried over Na_2SO_4, and the solvent was removed under reduced pressure to afford the stapled peptide *hS*$_{3,7}$*hS*-2 (61 mg, 88%), which was used for the subsequent reaction without further purification. A solution of the above peptide, *N*-methylmorpholine-*N*-oxide (NMO, 20 mg, 0.17 mmol), and 4% aqueous osmium tetroxide (25 μL) was dissolved in acetone/*t*-BuOH/water (7 mL, 3/3/1) and then stirred at room temperature for 24 h. Next, saturated aqueous sodium thiosulfate (30 mL) was added to the solution and stirred at room temperature for 30 min. The resultant solution was diluted with AcOEt, washed with 1% aqueous HCl and brine, and dried over Na_2SO_4. After the solvent had been removed, the residue was purified by column chromatography on silica gel (AcOEt/MeOH = 20:1) to give ***hS*$_{3,7}$*hS*^{dOH}-2** (39 mg, 62%). Spectroscopic data for ***hS*$_{3,7}$*hS*^{dOH}-2**: Foam; $[\alpha]_D^{24}$ −33.9 (*c* 0.50, $CHCl_3$); IR (in $CHCl_3$): ν = 3326, 2959, 1665, 1529, 1251, 1159 $^{cm-1}$; ^1H NMR (400 MHz, $CDCl_3$) 7.96 (d, *J* = 6.4 Hz, 1H), 7.56 (d, *J* = 4.8 Hz, 1H), 7.26 (m, 2H), 7.16 (d, *J* = 10.0 Hz, 1H), 6.98 (d, *J* = 4.8 Hz, 1H), 6.56 (m, 1H), 4.99–5.12 (m, 2H), 4.50–4.78 (m, 2H), 4.25 (m, 1H), 4.13 (m, 1H), 3.94–4.02 (m, 3H), 3.73 (s, 3H), 3.54–3.68 (m, 6H), 3.22–3.46 (m, 3H), 2.22 (m, 1H), 1.81–1.94 (m, 4H), 1.61–1.71 (m, 10H), 1.43–1.48 (m, 14H), 1.00–1.30 (m, 2H), 0.89–0.99 (m, 24H); [HR-ESI(+)]: *m*/*z* calcd for $C_{46}H_{83}N_7O_{14}Na$ $[M+Na]^+$: 980.5890; actual: 980.5883.
33. A solution of ***hS*$_{3,7}$*hS*^{dOH}-2** (30 mg, 0.03 mmol) and 1 M aqueous NaOH (0.06 mL) in MeOH (1 mL) was stirred at room temperature for 12 h. Then, the solution was neutralized with 1 M aqueous HCl, extracted with AcOEt, and dried over Na_2SO_4. After the solvent had been removed, the residue was purified by column chromatography on silica gel (AcOEt/MeOH = 5:1) to give ***hS*$_{3,7}$*hS*^{dOH}-2**-carboxylic acid. Next, 6 M aqueous HCl (0.05 mL) was added to a solution of the above acid in tetrahydrofuran (1 mL) at 0 °C, before being stirred at room temperature for 5 h. The removal of the solvent afforded the N- and C-terminal-free heptapeptide **H-*hS*$_{3,7}$*hS*^{dOH}-2-OH** (**DPI-07**) (20 mg, 81%). Spectroscopic data for **DPI-07**: Foam; $[\alpha]_D^{24}$ −16.1 (*c* 1.00, $CHCl_3$); ^1H NMR (400 MHz, CD_3OD) δ 4.68 (m, 1H), 4.20–4.48 (m, 4H), 3.96 (m, 1H), 3.48–3.81 (m, 10H), 1.47–2.39 (m, 22H), 0.95–1.06 (m, 24H); [HR-ESI(+)]: *m*/*z* calcd for $C_{40}H_{74}N_7O_{12}$ $[M+H]^+$: 844.5395; actual: 844.5350.
34. Synthesized peptides were dissolved in DMSO to final concentrations of 2.5×10^{-6}, 2.5×10^{-5}, 2.5×10^{-4}, and 2.5×10^{-3} M. The relative activity of the positive control $1\alpha,25(OH)_2D_3$ was calculated the formula $B/B_{max} = (C - B)/(A - B)\%$, where *A* is the OD_{450} value of the positive control in an SRC(+) well minus the OD_{450} value of the positive control in an SRC(−) well; *B* is the OD_{450} value of the negative control in an SRC(+) well minus the OD_{450} value of the positive control in an SRC(−) well; *C* is the OD_{450} value of the peptide sample in an SRC(+) well minus the OD_{450} value of the peptide sample in an SRC(−) well. All peptides were precipitated in 2.5×10^{-3} M.

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36. The helicity of stapled peptides was higher than that of the corresponding linear peptides. Furthermore, the inhibitory activity of stapled peptides showed stronger than that of the linear peptides (for instance, 220 μM for the stapled peptide **H-*hS*$_{3,7}$*hS*-2-OH**, 500 μM for the corresponding linear peptide **H-*hS*$_{3,7}$*hS*-1-OH**).
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38. The N- and C-termini of **DPI-07** are capped with *N*-acetyl and methylamide groups, respectively. It was determined that **DIP-07** exists as a mixture of two stereoisomers.

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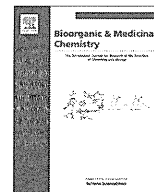

Synthesis and evaluation of novel 3-(3,5-dimethylbenzyl)uracil analogs as potential anti-HIV-1 agents

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A novel series of uracil derivatives with a 3,5-dimethylbenzyl group at the N^3-position were synthesized and evaluated as non-nucleoside HIV-1 reverse transcriptase inhibitors. Some of these compounds showed good-to-moderate activity with EC_{50} values in the submicromolar range. Among them, compound **10c** showed significant potency against HIV-1 activity with an EC_{50} value of 0.03 μM and a high selectivity index of 2863. Preliminary structure–activity relationships and molecular modeling analyses were used to explore the major interactions between HIV-1 reverse transcriptase and the potent inhibitor **10c**, which may serve as an important lead for further optimization.

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1. Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a structurally diverse group of compounds that bind to the viral enzyme reverse transcriptase (RT), where the NNRTIs[1] interact with a specific allosteric non-substrate binding pocket site. It is interesting that some NNRTIs have an aromatic group at the 6-position of uracil skeleton. For instance, in 1989, Baba et al.[2a] discovered that 1-[(2-hydroxyethoxy)methyl]-6-phenylthiothymine (**1a**, HEPT, Fig. 1) had strong anti-HIV-1 activity. Interestingly, HEPT **1a** can be regarded as an acyclonucleoside analog, yet its 5′-triphosphate derivative **1b** showed no inhibitory effect on HIV-1 RT,[2b] suggesting that HEPT could be undoubtedly considered an NNRTI despite having a uracil skeleton. Since the first finding of HEPT **1a**, related uracil derivatives have been synthesized.[2] We have consistently searched for an anti-HIV-1 agent using the structure–activity relationships (SAR) of the 1,3-disubstituted and 1,3,6-trisubstituted uracils.[3] As a result, we have demonstrated that the 3,5-dimethylbenzyl group at the 3-position of the uracil skeleton plays an important role in an enhancement of the anti-HIV-1 activity; notably, 1-substituted (e.g., benzyl, cyanomethyl, or 4-picolyl group) 3-(3,5-dimethylbenzyl)uracil **2a–c** showed good antiviral activity.[3a] Moreover, the introduction of an azido or amino group at the 6-position of 1-benzyl-3-(3,5-dimethylbenzyl)uracil **2d** and **2e** showed excellent potency with an EC_{50} of 0.067 ± 0.011 μM and 0.069 ± 0.006 μM, respectively.[3c] However, the CC_{50} and corresponding SI values of compounds **2d** and **2e** were not satisfactory (CC_{50} 45.9 ± 0.7 μM and 45.6 ± 0.9 μM; SI 685 and 661, respectively).[3c] Taken together, these results prompted us to evaluate the following newly synthesized 1,3,6-trisubstituted uracils with an aim to develop higher anti-HIV-1 activity and lower cytotoxicity: (i) 6-alkylamino-introduced analogs of 1-benzyl-3-(3,5-dimethylbenzyl)uracil (**5 series**) to examine the effect of N-alkylation of the C6-amino group on **2e**, and (ii) 1-substituted derivatives of 6-azido (or amino)-3-(3,5-dimethylbenzyl)uracil (**9** and **10 series**) to investigate substituent effect when the N^1-benzyl group of **2d** or **2e** is replaced by an appropriate alkyl group such as cyanomethyl, 2-picolyl and 4-picolyl groups. We also report on the docking studies of the most promising inhibitor **10c** with an RT nevirapine binding site.

2. Results and discussion

2.1. Chemistry

To obtain a series of 6-amino-substituted analogs of 1-benzyl-3-(3,5-dimethylbenzyl)uracil (**5 series**), 1-benzyl-6-chloro-3-(3,5-dimethylbenzyl)uracil **4** was prepared in two steps from the commercially available 6-chlorouracil **3** using our previously

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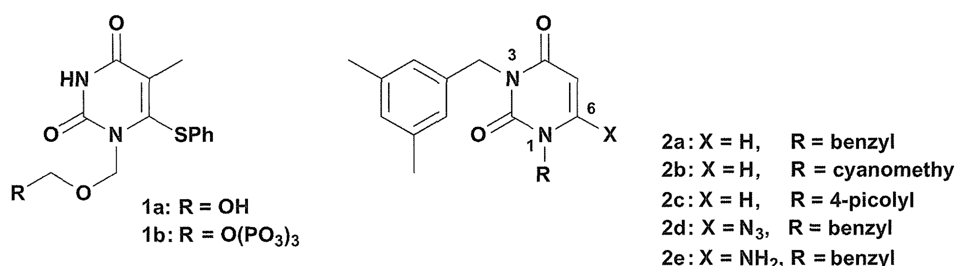


Figure 1. Structures of HEPT derivatives (**1a**, **b**) and 1-substituted-3-(3,5-dimethylbenzyl)uracil derivatives (**2a–e**).

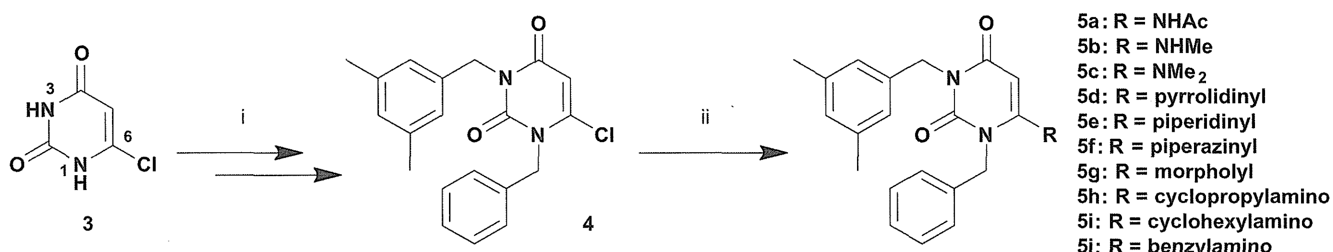
reported method shown in Scheme 1.[3c] Then, compound **4** was subjected to nucleophilic substitution with a variety of acyclic or cyclic amines (e.g., dimethylamine, pyrrolidine, morpholine, or benzylamine) in the presence of Na_2CO_3 in EtOH at 50–100 °C for 0.5–65 h to give their 6-alkylamino-substituted analogs in 65–100% yields (Scheme 1, **5b–j**). In the case of the 6-acetamide analog **5a**, 6-amino derivative **2b** prepared from **3**[3c] was N-monoacetylated with Ac_2O and pyridine to obtain the corresponding derivative **5a**.

The 6-azido (and amino)-1-substituted analogs of 3-(3,5-dimethylbenzyl)uracil synthesis (**9** and **10 series**) also began with 6-chlorouracil **4** as a starting material and led to 1-methoxymethyl-6-chlorouracil **6** (Scheme 2). The resulting **6** was condensed with 3,5-dimethylbenzyl alcohol using the Mitsunobu reaction[3c] to afford 3-(3,5-dimethybenzyl) congener **7**. The product was carried out by deprotection of the N^1-MOM group using B-bromocatecholborane[4] in CH_2Cl_2 to give the corresponding N^1-deprotected inseparable mixture of **8a** and 6-bromo-substituted product **8b** in 72% combined yield (ratio **8a**/**8b**: 80/20). Next, the resulting mixture of **8a** and **8b** was treated with bromoacetonitrile, 2-picolyl chloride or 4-picolyl chloride to give the N^1-alkylated congeners, and subsequently, the mixture of 6-chloro and 6-bromo derivatives was subjected to the nucleophilic substitution at the C6-position in the uracil moiety with sodium azide to afford the single product of 6-azido substituted analogs **9a–c**. The reduction of compounds **9b** and **9c** was carried out with lithium aluminum hydride to provide 6-amino compound **10b** and **10c**, whereas the conversion of **9a** was performed using PPh_3–H_2O condition[5] to obtain **10a**, avoiding the excessive reduction of cyanomethyl group of **9a**.

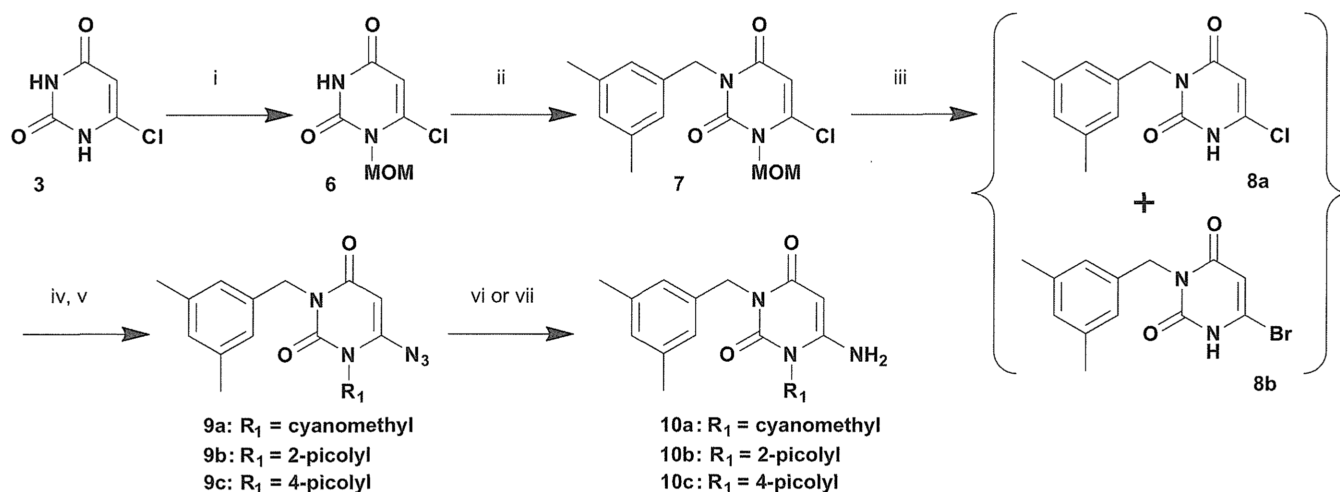
2.2. Biological activity

The antiviral activity of the 6-alkylamino-substituted 1-benzyl-3-(3,5-dimethylbenzyl)uracils **5a–j**, 6-azido congeners **9a–c**, and 6-amino analogs **10a–c** of 1-substituted-3-(3,5-dimethylbenzyl)uracils was determined by examining the inhibitory effects of these compounds on the HIV-1-induced cytopathogenicity and on cell viability in MT-4 cells. Zidovudine (AZT) was also tested as a positive control. In the series of 6-alkylamino-substituted analogs **5a–j** shown in Table 1, almost every compound had considerably reduced anti-HIV-1 activity when compared with 6-azido or amino-introduced derivatives **2d** and **2e**, which displayed EC_{50} values of 0.067 ± 0.011 μM and 0.069 ± 0.006 μM, respectively,[3c] suggesting that alkylation of the amino group at the 6-position on the uracil moiety diminished anti-HIV-1 activity. Even N-monomethylation of the amino group at the 6-position, such as for compound **5b**, resulted in centesimal decreased HIV-1 activity (EC_{50} = 7 ± 4 μM) in comparison to the corresponding 6-free amino analog **2e**.

Next, the introduction of an alkyl group (such as cyanomethyl, 2-picolyl, and 4-picolyl) at the N^1-position of the uracil skeleton in place of N^1-benzyl group of **2d** and **2e** was investigated for the SAR. Among the compounds **9a–c** and **10a–c** shown in Table 1, N^1-2-picolyluracil congeners **9b** and **10b** showed good anti-HIV-1 activity with EC_{50} values of 0.22 ± 0.10 μM and 0.23 ± 0.06 μM, respectively. Because CC_{50} values for both **9b** and **10b** exceeded 100 μM, their SI values were >457 and >443, respectively, which were comparable to the SI values of the N^1-benzyl uracil derivatives **2d** and **2e** (658 and 661, respectively), reported in our previous study.[3c] However, the introduction of an N^1-4-picolyl group in **9c** and **10c** produced compounds with the strongest anti-HIV-1 potencies (EC_{50} 0.05 ± 0.02 μM and 0.03 ± 0.03 μM, respectively). In particular, the 6-amino-1-(4-picolyl) analog **10c** not only exhibited significant activity (at EC_{50} = 0.03 ± 0.03 μM), but also displayed low cytotoxicity with CC_{50} >100 μM, resulting in an SI >2863, which is high and comparable to those of the positive controls AZT (SI >2769) or nevirapine[3c] (SI >1639). In contrast, the 6-azido-1-(4-picolyl) congener **9c** showed moderate values for CC_{50} of 59 ± 6 μM and for SI of 1162. As for the similar potencies (defined by their similar EC_{50} values) among C6-azido analogs **2d**, **9b** and **9c**, and C6-amino derivatives **2e**, **10b** and **10c**, it is possible that 6-azidouracil developed antiviral activity after metabolic conversion to the 6-aminocongener. Finally, C6-azidouracil **9a** with a cyanomethyl group at the N^1 position was, unfortunately, 100 times less potent (EC_{50} = 3.5 ± 1 μM) than its N^1-4-picolyl counterpart, **10c**. Moreover, the 6-amino-1-cyanomethyl analog **10a** showed no antiviral activity.

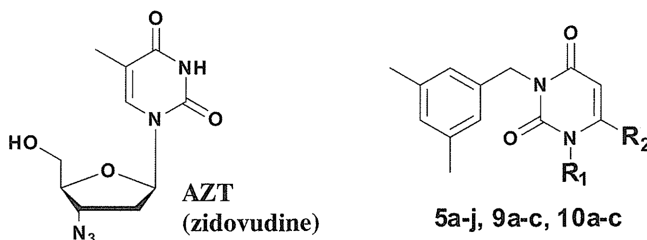


Scheme 1. Synthesis of 6-alkylamino-substituted-1-benzyl-3-(3,5-dimethylbenzyl)uracil (**5 series**). Reagents and conditions: (i) see Ref. 3c; (ii) K_2CO_3, EtOH, 50–100 °C, 0.5–65 h, 65–100%.



Scheme 2. Synthesis of 6-azido-substituted-1-benzyl-3-(3,5-dimethylbenzyl)uracil (**9 series**) and Synthesis of 6-amino-1-substituted-3-(3,5-dimethylbenzyl)uracil (**10 series**). Reagents and conditions: (i) MOMCl, DBU, CH_2Cl_2, 0 °C, 20 min, 90%; (ii) 2,6-dimethylbenzylalcohol, TMAD, PPh_3, 50 °C, 2 h, 93%; (iii) B-bromocatecholborane, CH_2Cl_2, rt, 1 h, 72%; (iv) R-X (R = cyanomethyl, X = Br; R = 2-picolyl, X = Cl), K_2CO_3, DMF, 40–93%; (v) NaN_3, DMF, rt, 30 min, 81–90%; (vi) PPh_3, H_2O, THF, rt, 10 h, 90%; (vii) $LiAlH_4$, THF, 0 °C, 5 min, 69–74%.

Table 1
Antiviral activity of 3-(3,5-dimethylbenzyl)uracil analogs against HIV-1



Compound	R_1	R_2	EC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
AZT	–	–	0.04 ± 0.04	>100	>2769
5a	Bn	NHAc	>100	>100	–
5b	Bn	NHAc	7 ± 4	65 ± 3	9
5c	Bn	NMe_2	>48 ± 5	48 ± 5	–
5d	Bn	Pyrrolidinyl	>31 ± 3	31 ± 3	–
5e	Bn	Piperidinyl	>47 ± 3	47 ± 3	–
5f	Bn	Piperidinyl	>16 ± 4	16 ± 4	–
5g	Bn	Morpholyl	>100	>100	–
5h	Bn	Cyclopropylamino	>100	>100	–
5i	Bn	Cyclohexylamino	>74 ± 19	74 ± 19	–
5j	Bn	NHBn	>100	>100	–
9a	Cyanomethyl	N_3	3.5 ± 1	60 ± 1	–
9b	2-Picolyl	N_3	0.22 ± 0.10	>100	17
9c	4-Picolyl	N_3	0.05 ± 0.02	59 ± 6	>457
10a	Cyanomethyl	NH_2	>100	>100	1162
10b	2-Picolyl	NH_2	0.23 ± 0.06	>100	>443
10c	4-Picolyl	NH_2	0.03 ± 0.03	>100	>2863

[a] EC_{50}, effective concentration; the concentration of compound required to protect the cell against viral cytopathogenicity by 50% in MT-4 cells.
[b] CC_{50}, cytotoxic concentration; the concentration of compound that reduces the normal uninfected MT-4 cells viability by 50%.
[c] SI, selectivity index (CC_{50}/EC_{50}).

2.3. Molecular modeling analysis

The X-Ray co-crystal structure (PDB: 1VRT) of HIV-1 RT with nevirapine was taken from PDB (1VRT)[6] and used for docking studies. A docking model of ligand **10c**, which showed the most promising HIV-1 activity, bound to HIV-1 RT was constructed by conformational search using *MacroModel* (ver. 9.1). AMBER* was used as a force field, and more than 3000 conformers for **10c** were optimized. The most stable conformation is shown in Figure 2. The hydrogen of the 6-amino group for **10c** is hydrogen bonded to the amide group of Lys101 residue (NH···O=C), and the 3,5-dimethylbenzyl moiety was oriented around the hydrophobic area (Tyr181, Tyr188, Trp229, and Leu234 residues) of HIV-1 RT. This result was significantly different from that of the C6-deaminated counterpart **2c** in our previous report.[3b] The nitrogen of the 4-picolyl formed a hydrogen bond with the amide group of the Lys101 residue (N···H–N), whereas the 3,5-dimethylbenzyl group at the N^3 position existed around another hydrophobic area (Val106, Pro225,

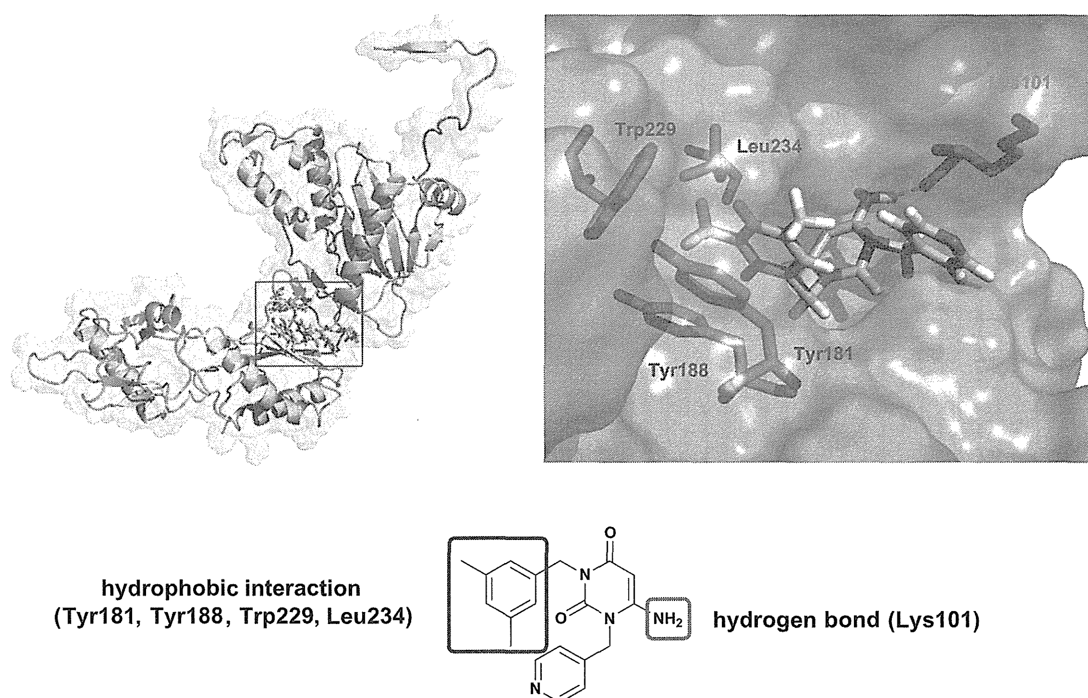


Figure 2. Docking structure between HIV-RT and 6-amino-3-(3,5-dimethylbenzyl)-1-(4-picolyl)uracil (**10c**).

Phe227, Leu234, and Pro236 residues) of HIV-1 RT. Most NNRTIs are known to be engaged in the hydrogen bond with the backbone of the amino acids Lys101.[7] Thus, the results from our docking study with compound **10c** suggest a role for the hydrogen bond in the affinity with RT.

2.4. Conclusion

In the present study, a series of newly synthesized 1-substituted analogs of 6-azido or 6-amino-3-(3,5-dimethylbenzyl)uracil exhibited good potency against HIV-1 activities. In particular, compound **10c** displayed excellent activity with an EC_{50} value of 0.03 ± 0.03 μM and an SI value of 2863. Furthermore, the simulated binding model of **10c** with HIV-1 RT indicated that the hydrogen of the 6-amino group for **10c** is hydrogen bonded to the amide group of the Lys101 residue, and that the 3,5-dimethylbenzyl moiety was oriented around the hydrophobic area (Tyr181, Tyr188, Trp229, and Leu234 residues) of HIV-1 RT. Overall, this compound may serve as the basis for further modification in the search for more potent candidates for anti-HIV-1 chemotherapy.

3. Experimental section

3.1. Chemistry

^{1}H NMR and ^{13}C NMR spectra were taken with an Ultrashield™ 400 Plus FT NMR System (BRUKER, Germany). Chemical shifts and coupling constants (*J*) were given in *δ* and Hz, respectively. Melting points were determined on a Yanaco MP-500D. High-resolution mass spectrometry was performed on an APEX IV mass spectrometer (BRUKER) with electrospray ionization mass spectroscopy (ESI-MS).

3.2. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-acetamidouracil (5a)

A solution of compound **2e** (33.5 mg, 0.1 mmol) and Ac_2O (189.1 μl, 2.0 mmol) in dry pyridine (0.3 ml) was stirred for 7 days at room temperature. The mixture was then evaporated in vacuo and the residue was extracted with AcOEt. The organic extracts were washed with water and saturated sodium chloride solution, dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (50% AcOEt in hexane) to a white crystal **5a** (13.2 mg, 0.035 mmol, 35%). ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 11.60 (1H, br s, AcNH), 7.21–7.40 (5H, m, Bn), 7.06 (2H, s, 3,5-Me_2-Bn), 6.90 (1H, s, 3,5-Me_2-Bn), 5.40 (1H, s, H-5), 5.21 (2H, s, Bn), 5.13 (2H, s, 3,5-Me_2-Bn), 2.64 (6H, s, Ac), 2.30 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 199.4, 161.3, 158.2, 150.4, 138.0, 136.9, 133.7, 129.6, 129.2, 128.7, 126.1, 126.1, 91.9, 46.1, 44.6, 32.5, 21.3; HRMS (ESI) Calcd for $C_{22}H_{23}N_3NaO_3^+$ $[M+Na]^+$: 400.16316. Found 400.16349; mp: 212.3 °C.

3.3. General procedure for the synthesis of 5b–j

A solution of compound **4** (177.4 mg, 0.5 mmol), appropriate amine (1.0 mmol) and Na_2CO_3 (106.0 mg, 1.0 mmol), in dry EtOH (3.0 ml) was heated at 50–100 °C. After 0.5–65 h stirring, the mixture was evaporated in vacuo and the residue was extracted with AcOEt. The organic extracts were washed with water and saturated sodium chloride solution, dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography to afford **5b–j** in 65–100% yield.

3.3.1. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-methylaminouracil (5b)

Yield 92%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 7.20–7.39 (5H, m, Bn), 7.08 (2H, s, 3,5-Me_2-Bn), 6.88 (1H, s, 3,5-Me_2-Bn), 5.14 (2H, s, 3,5-Me_2-Bn), 5.10 (2H, s, Bn), 4.88 (1H, s, H-5), 4.17 (1H, m, NH), 2.69 (3H, d, *J* 5.2, methylamino), 2.30 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* 162.7, 154.0, 152.1, 137.8, 137.5, 135.1, 129.3, 128.9, 128.2, 126.1, 126.0, 76.0, 45.7, 44.2, 29.9, 21.3; HRMS (ESI) Calcd for $C_{21}H_{23}N_3NaO_2^+$ $[M+Na]^+$: 372.16825. Found 372.16855; mp: 186.7 °C.

3.3.2. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-dimethylaminouracil (5c)

Yield 100%; pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.15–7.31 (5H, m, Bn), 6.99 (2H, s, 3,5-Me_2-Bn), 6.85 (1H, s, 3,5-Me_2-Bn), 5.29 (1H, s, H-5), 5.07 (2H, s, Bn), 5.00 (2H, s, 3,5-Me_2-Bn), 2.68 (6H, s, 6-NMe_2), 2.25 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.9, 160.6, 152.9, 137.7, 137.1, 136.9, 129.1, 128.6, 127.5, 126.8, 126.3, 88.4, 48.8, 44.1, 42.5, 21.3; HRMS (ESI) Calcd for $C_{22}H_{26}N_3O_3^+$ $[M+H]^+$:364.20195. Found 364.20256.

3.3.3. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-pyrrolidinyluracil (5d)

Yield 84%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.12–7.30 (5H, m, Bn), 7.04 (2H, s, 3,5-Me_2-Bn), 6.86 (1H, s, 3,5-Me_2-Bn), 5.21 (1H, s, H-5), 5.10 (2H, s, 3,5-Me_2-Bn), 5.04 (2H, s, Bn), 3.13 (4H, m, pirrolidinyl), 2.26 (6H, s, 3,5-Me_2-Bn), 1.86 (4H, m, pirrolidinyl); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.8, 157.2, 153.3, 137.7, 137.3, 136.9, 129.0, 128.6, 127.3, 126.4, 126.0, 84.0, 51.5, 49.9, 44.1, 25.3, 21.3; HRMS (ESI) Calcd for $C_{24}H_{27}N_3NaO_2^+$ $[M+Na]^+$: 412.19955. Found 412.19893; mp: 132.2 °C.

3.3.4. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-piperidinyluracil (5e)

Yield 96%; pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.19–7.29 (5H, m, Bn), 7.00 (2H, s, 3,5-Me_2-Bn), 6.86 (1H, s, 3,5-Me_2-Bn), 5.30 (1H, s, H-5), 5.04 (2H, s, 3,5-Me_2-Bn), 5.00 (2H, s, Bn), 2.85 (4H, m, piperidinyl), 2.24 (6H, s, 3,5-Me_2-Bn), 1.56 (6H, m, piperidinyl); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.1, 160.6, 152.9, 137.8, 137.8, 137.1, 129.1, 128.6, 127.5, 127.0, 126.4, 89.7, 52.3, 48.1, 44.1, 25.3, 23.9, 21.3; HRMS (ESI) Calcd for $C_{25}H_{29}N_3NaO_2^+$ $[M+Na]^+$: 426.21520. Found 426.21293.

3.3.5. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-piperazinyluracil (5f)

Yield 92%; pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.19–7.32 (5H, m, Bn), 7.01 (2H, s, 3,5-Me_2-Bn), 6.86 (1H, s, 3,5-Me_2-Bn), 5.33 (1H, s, H-5), 5.07 (2H, s, Bn), 5.01 (2H, s, 3,5-Me_2-Bn), 2.93 (4H, s, piperazinyl), 2.87 (4H, s, piperazinyl), 2.25 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.9, 160.0, 152.8, 137.8, 136.9, 129.1, 128.7, 127.6, 126.8, 126.3, 90.1, 52.2, 48.1, 45.3, 44.2, 21.3; HRMS (ESI) Calcd for $C_{24}H_{29}N_4NaO_2^+$ $[M+Na]^+$:405.22850. Found 405.22688.

3.3.6. 1-Benzyl-3-(3,5-dimethylbenzyl)-6-morpholyluracil (5g)

Yield 100%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.24–7.33 (5H, m, Bn), 7.02 (2H, s, 3,5-Me_2-Bn), 6.87 (1H, s, 3,5-Me_2-Bn), 5.35 (1H, s, H-5), 5.09 (2H, s, Bn), 5.02 (2H, s, 3,5-Me_2-Bn), 3.74 (4H, s, morpholyl), 2.88 (4H, s, morpholyl), 2.25 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.8, 159.5, 152.7, 137.8, 136.9, 136.8, 129.2, 128.8, 127.7, 126.7, 126.4, 90.3, 66.1, 51.4, 48.1, 44.2, 21.3; HRMS (ESI) Calcd for $C_{24}H_{27}N_3NaO_3^+$ $[M+Na]^+$:428.19446. Found 428.19306; mp: 133.2 °C.

3.3.7. 1-Benzyl-6-cyclopropylamino-3-(3,5-dimethylbenzyl)uracil (5h)

Yield 94%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26–7.38 (5H, m, Bn), 7.10 (2H, s, 3,5-Me_2-Bn), 6.89 (1H, s, 3,5-Me_2-Bn), 5.31 (1H, s, H-5), 5.10 (2H, s, Bn), 5.08 (2H, s, 3,5-Me_2-Bn), 4.46 (1H, br s, NH), 2.29 (7H, m, 3,5-Me_2-Bn and cyclopropylamino), 0.42–0.75 (4H, m, cyclopropylamino); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.2, 154.1, 151.5, 137.9, 137.1, 136.5, 128.4, 128.2, 127.1, 126.1, 124.9, 75.5, 44.3, 42.9, 24.2, 20.9, 6.6; HRMS (ESI) Calcd for $C_{23}H_{25}N_3NaO_2^+$ $[M+Na]^+$: 398.18390. Found 398.18193; mp: 214.4 °C.

3.3.8. 1-Benzyl-6-cyclohexylamino-3-(3,5-dimethylbenzyl)uracil (5i)

Yield 89%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.22–7.34 (5H, m, Bn), 7.10 (2H, s, 3,5-Me_2-Bn), 6.88 (1H, s, 3,5-Me_2-Bn), 5.16 (2H, s, 3,5-Me_2-Bn), 5.10 (2H, s, Bn), 4.88 (1H, s, H-5), 4.13 (1H, m, cyclohexylamino), 2.30 (6H, s, 3,5-Me_2-Bn), 2.05 (1H, br s, NH), 1.53-1.76 (4H, m, cyclohexylamino), 1.11–1.47 (6H, m, cyclohexylamino); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.8, 152.4, 151.9, 137.8, 137.6, 135.3, 129.4, 129.0, 128.4, 126.3, 126.2, 76.2, 51.3, 46.0, 44.2, 31.9, 25.3, 24.0, 21.3; HRMS (ESI) Calcd for $C_{26}H_{31}N_3NaO_2^+$ $[M+Na]^+$: 440.23085. Found 440.22996; mp: 71.8 °C.

3.3.9. 1-Benzyl-6-benzylamino-3-(3,5-dimethylbenzyl)uracil (5j)

Yield 64%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.26–7.36 (5H, m, Bn), 7.18–7.19 (2H, s, 3,5-Me_2-Bn), 6.89–6.95 (5H, m, benzylamino), 5.18 (2H, s, Bn), 5.10 (2H, s, 3,5-Me_2-Bn), 4.92 (1H, s, H-5), 4.46 (1H, m, NH), 4.11 (2H, d, J 5.2, benzylamino), 2.29 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.7, 152.7, 152.2, 137.8, 137.4, 135.9, 135.2, 129.3, 129.0, 128.9, 128.3, 128.0, 127.1, 126.3, 126.2, 76.8, 47.1, 45.8, 44.2, 21.3; HRMS (ESI) Calcd for $C_{27}H_{27}N_3NaO_2^+$ $[M+Na]^+$: 448.19955. Found 448.19661; mp: 189.1 °C.

3.4. 6-Chloro-1-methoxymethyluracil (6)

6-Chlorouracil (**3**) (1.46 g, 10.0 mmol) was dissolved in dry CH_2Cl_2 (30 ml), and DBU (1646.3 µl, 11.0 mmol) was added to the solution at room temperature, and after stirring an additional 10 min, the mixture became clear. After the mixture was cooled to 0 °C, methyl chloromethyl ether (911.1 µl, 12.0 mmol, MOMCl) was added dropwise, and stirring continued for an additional 20 min at 0 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (AcOEt) to white powder **6** (1.71 g, 8.99 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (1H, s, NH), 5.94 (1H, s, 5-H), 5.43 (2H, s, MOM), 3.47 (3H, s, MOM).

3.5. 6-Chloro-1-methoxymethyl-3-(3,5-dimethylbenzyl)uracil (7)

A solution of compound **6** (1.44 g, 7.56 mmol), triphenylphosphine (2.58 g, 9.83 mmol), 3,5-dimethylbenzylalcohol (1.17 ml, 7.94 mmol) and TMAD (*N,N,N′,N′*-tetramethylazodicarboxamide, 1.69 g, 9.83 mmol) in THF (28.0 ml) was stirred at 50 °C. After 2 h stirring, the solution was filtered and concentrated to a small volume. The residual solution was purified by silica gel column chromatography (30% AcOEt in hexane) to give as a syrup **7** (2.17 g, 7.03 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.04 (2H, s, 3,5-Me_2-Bn), 6.90 (1H, s, 3,5-Me_2-Bn), 5.98 (1H, s, 5-H), 5.44 (2H, s, MOM), 5.03 (2H, s, 3,5-Me_2-Bn), 3.44 (3H, s, MOM), 2.28 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.5, 151.4, 145.2, 138.0, 136.0, 129.5, 126.6, 103.3, 57.5, 44.8, 21.3; HRMS (ESI) Calcd for $C_{15}H_{17}ClN_2NaO_3$ $[M+Na]^+$: 331.08199. Found 331.08154.

3.6. 6-Chloro-3-(3,5-dimethylbenzyl)uracil (8a) and 6-bromo-3-(3,5-dimethylbenzyl)uracil (8b)[4]

A solution of compound **7** (1.90 g, 6.15 mmol) and B-bromocatecholborane (1.47 g, 7.38 mmol, 0.2 M in CH_2Cl_2) in CH_2Cl_2 (20 ml) was stirred at room temperature. After 2 h stirring, the mixture was evaporated in vacuo, and the residue was extracted with AcOEt. The organic extracts were washed with saturated aqueous $NaHCO_3$ and saturated NaCl solution, dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (40% AcOEt in hexane) to give the inseparable mixture of **8a** and **8b** as a needle crystal (1.21 g, 4.42 mmol, 72% combined yield), which was employed in the next reaction without further purification, in a ratio of 80:20 according to ^{1}H NMR spectrum. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.06 (2H, s, 3,5-Me_2-Bn), 6.90 (1H, s, 3,5-Me_2-Bn), 6.05 (0.2H, s, 5-H, **8b**), 5.89 (0.8H, s, 5-H, **8a**), 4.99 (2H, s, 3,5-Me_2-Bn), 2.27 (6H, s, 3,5-Me_2-Bn); HRMS (ESI) Calcd

for $C_{13}H_{13}ClN_2NaO_2$ [M+Na]$^+$: 287.05578. Found 278.05656 (**8a**), Calcd for $C_{13}H_{13}BrN_2NaO_2$ [M+Na]$^+$: 331.00526. Found 331.00300 (**8b**).

3.7. General procedure for the synthesis of 9a–c

The mixture of **8a** and **8b** (264.7 mg, 0.97 mmol) was dissolved in dry DMF (5.0 ml) under nitrogen atmosphere. To this stirred solution, we carefully added the appropriate alkyl halide (bromoacetonitrile, 2-(chloromethyl)pyridine hydrochloride or 4-(chloromethyl)pyridine hydrochloride; 2.0 mmol) and K_2CO_3 (276.4 mg, 2.0 mmol). Stirring was continued at room temperature for 2–12 h, and the mixture was extracted with AcOEt. The organic extracts were washed with water and saturated aqueous sodium chloride solution, dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography to give the inseparable mixture of C6-chloro and C6-bromo congeners, ratio 80:20 according to ^{1}H NMR spectrum.

The resulting mixture (258.1 mg, 0.83 mmol) was dissolved in dry DMF (5.0 ml), and NaN_3 (66.3 mg, 1.02 mmol) was added to the solution, which was stirred for 30 min at room temperature. The mixture was extracted with AcOEt, washed with saturated aqueous sodium chloride solution, dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (70% AcOEt in hexane) to give C6-azido derivative **9a–c**.

3.7.1. 6-Azido-1-cyanomethyl-3-(3,5-dimethylbenzyl)uracil (9a)

Combined yield 82%; brown oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.04 (2H, s, 3,5-Me_2-Bn), 6.91 (1H, s, 3,5-Me_2-Bn), 5.64 (1H, s, 5-H), 5.02 (2H, s, 3,5-Me_2-Bn), 4.75 (2H, s, cyanomethyl), 2.28 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.5, 150.0, 148.9, 138.1, 135.8, 129.6, 126.6, 113.8, 88.9, 44.9, 30.8, 21.3; HRMS (ESI) Calcd for $C_{15}H_{15}N_6NaO_2$ [M+Na]$^+$: 333.10704. Found 333.10687.

3.7.2. 6-Azido-3-(3,5-dimethylbenzyl)-1-(2-picolyl)uracil (9b)

Combined yield 32%; brown oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.54 (1H, d, *J* 4.0, 2-picolyl), 7.66 (1H, m, 2-picolyl), 7.20 (2H, m, 2-picolyl), 7.02 (2H, s, 3,5-Me_2-Bn), 6.88 (1H, s, 3,5-Me_2-Bn), 5.61 (1H, s, 5-H), 5.17 (2H, s, 2-picolyl), 5.05 (2H, s, 3,5-Me_2-Bn), 2.27 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.4, 154.9, 151.2, 151.2, 149.6, 137.9, 136.7, 136.5, 129.2, 126.2, 122.6, 121.1, 88.0, 48.0, 44.5, 21.2; HRMS (ESI) Calcd for $C_{19}H_{18}N_6NaO_2$ [M+Na]$^+$: 385.13834. Found 385.13740.

3.7.3. 6-Azido-3-(3,5-dimethylbenzyl)-1-(4-picolyl)uracil (9c)

Combined yield 59%; brown oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58 (2H, d, *J* 6.0, 4-picolyl), 7.16 (2H, d, *J* 5.6, 4-picolyl), 7.03 (2H, s, 3,5-Me_2-Bn), 6.90 (1H, s, 3,5-Me_2-Bn), 5.60 (1H, s, 5-H), 5.06 (2H, s, 4-picolyl), 5.03 (2H, s, 3,5-Me_2-Bn), 2.28 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.0, 151.1, 150.4, 150.3, 144.6, 138.0, 136.3, 129.4, 126.4, 122.1, 88.2, 45.8, 44.7, 21.3; HRMS (ESI) Calcd for $C_{19}H_{18}N_6NaO_2$ [M+Na]$^+$: 385.13834. Found 385.13777.

3.8. 6-Amino-1-cyanomethyl-3-(3,5-dimethylbenzyl)uracil (10a)

A solution of compound **9a** (73.0 mg, 0.23 mmol), triphenylphosphine (74.0 mg, 0.28 mmol) and H_2O (6.2 µl, 0.35 mmol) in THF (1.0 ml) was stirred at room temperature. After stirring for 10 h, the solution was concentrated to a small volume. The residual solution was purified by silica gel column chromatography (20% MeOH in $CHCl_3$) to give as a yellowish crystal **10a** (60.0 mg, 0.21 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.98 (2H, s, 3,5-Me_2-Bn), 6.88 (1H, s, 3,5-Me_2-Bn), 5.07 (2H, br s, NH_2), 5.05 (1H, s, 5-H), 4.99 (2H, s, cyanomethyl), 4.80 (2H, s, 3,5-Me_2-Bn), 2.27 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 153.2, 150.8, 138.2, 136.6, 129.3, 125.6, 114.3, 78.4, 44.6, 30.4, 21.2; HRMS (ESI) Calcd for $C_{15}H_{16}N_4NaO_2$ [M+Na]$^+$: 307.11655. Found 307.11537; mp: 350.0 °C.

3.9. General procedure for the synthesis of 10b and 10c

Compound **9b** or **9c** (109.4 mg, 0.30 mmol) was dissolved in dry THF (5.4 ml) under nitrogen atmosphere. To this stirred solution we carefully added $LiAlH_4$ (13.7 mg, 0.36 mmol) at 0 °C. Stirring was continued at 0 °C for 5 min, and the reaction quenched by the addition of AcOEt (5.0 ml) until no effervescence was observed. Aqueous 1 N HCl (2.2 ml) was then added, and the product was extracted with AcOEt. The combined organic extracts were washed with water and saturated sodium chloride solution, dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (20% MeOH in $CHCl_3$) to give a white crystal **10b** or **10c**.

3.9.1. 6-Amino-3-(3,5-dimethylbenzyl)-1-(2-picolyl)uracil (10b)

Yield 69%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.48 (1H, d, *J* 4.0, 2-picolyl), 7.73 (1H, m, 2-picolyl), 7.61 (1H, d, *J* 8.0, 2-picolyl), 7.27 (1H, m, 2-picolyl), 7.03 (2H, s, 3,5-Me_2-Bn), 6.85 (1H, s, 3,5-Me_2-Bn), 6.21 (2H, br s, NH_2), 5.13 (2H, s, 2-picolyl), 5.03 (1H, s, 5-H), 5.01 (2H, s, 3,5-Me_2-Bn), 2.26 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.8, 155.5, 155.3, 152.0, 148.7, 138.0, 137.8, 137.4, 128.9, 126.2, 125.0, 123.6, 79.4, 48.7, 44.2, 21.3; HRMS (ESI) Calcd for $C_{19}H_{20}N_4NaO_2$ [M+Na]$^+$: 359.14785. Found 359.14714; mp: 189.3 °C.

3.9.2. 6-Amino-3-(3,5-dimethylbenzyl)-1-(4-picolyl)uracil (10c)

Yield 74%; white crystal; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.61 (2H, d, *J* 4.0, 4-picolyl), 7.14 (2H, d, *J* 8.0, 4-picolyl), 7.04 (2H, s, 3,5-Me_2-Bn), 6.88 (1H, s, 3,5-Me_2-Bn), 5.14 (2H, s, 4-picolyl), 5.06 (2H, s, 3,5-Me_2-Bn), 5.02 (1H, s, 5-H), 4.32 (2H, br s, NH_2) 2.28 (6H, s, 3,5-Me_2-Bn); ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.1, 156.9, 153.2, 150.3, 147.9, 138.9, 138.7, 129.7, 126.5, 122.9, 77.1, 45.8, 45.1, 21.4; HRMS (ESI) Calcd for $C_{19}H_{20}N_4NaO_2$ [M+Na]$^+$: 359.14785. Found 359.14734; mp: 142.0 °C.

3.10. Anti-HIV-1 assay

MT-4 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin G, and 100 mg/ml of streptomycin. The III_B strain of HIV-1 was used throughout the experiment. The virus was propagated and titrated in MT-4 cells. Virus stocks were stored at −80 °C until use. The anti-HIV-1 activity of the test compounds was determined by the inhibition of virus-induced cytopathogenicity in MT-4 cells.[8] Briefly, MT-4 cells (1×10^5 cells/ml) were infected with HIV-1 at a multiplicity of infection (MOI) of 0.1 and were cultured in the presence of various concentrations of the test compounds. After 4-day incubation at 37 °C in 5% CO_2, the number of viable cells was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method.[9] The cytotoxicity of the compounds was evaluated in parallel with their antiviral activity, based on the viability of mock-infected cells, as determined by the MTT method.

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Supplementary data

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target compounds, that were detected by LC-Q-TOF. All the substrates were extensively metabolized; main reactions involved N-dealkylation, hydroxylation on various molecules' moieties, oxygenation, reduction of the keto-group and/or dehydrogenation of the aliphatic chain, carboxylation and glucuronidation, the latter only for JWH251. The main typical mass fragments were identified for each metabolite. Based on the results obtained by Q-TOF, a LC-MS/MS method was set up for the routine identification of the main metabolites of these substances in biological fluids. The method was set up for the identification of N-dealkylated and hydroxylated metabolites on the side chain of each JWH, on indole group of JWH015, on fluoro-phenyl group of JWH307, on naphtyl group of JWH098; also demethyl, hydroxyl dimethyl and carbonyl JWH098, and carbonyl, dehydro and di-hydroxy JWH 307 have been included in the method, as well as dehydrogenated, reduced on carbonyle, carboxylated and glucuronated metabolites of JWH251. **Conclusions:** This study demonstrated the suitability of metasite and massmetasite programs for the prediction and identification of the metabolites of new chemical entities. The present work allowed to set up a method for the identification of main urinary metabolites of JWH015, 307, 251 and 098.

Keywords: Synthetic cannabinoids, Metabolites, In sylico metabolism, In vitro metabolism, HRMS, LC/MS

OE_{12}
TWO NEW DESIGNER DRUGS, MT-45 (I-C6) AND NOOPEPT (GVS-111), DETECTED WITH A SYNTHETIC CANNABINOID A-834735 IN ILLEGAL PRODUCTS

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Introduction: In this study, we identified three new designer drugs, a piperazine derivative MT-45 (I-C6), a synthetic peptide Noopept (GVS-111) and a synthetic cannabinoid A-834735, in illegal products distributed in Japan. **Materials and Methods:** One hundred forty analyzed samples were purchased via the Internet in 2013 as chemical or herbal products sold in Japan. The products were extracted with MeOH under ultrasonication. After centrifugation and filtration, the methanolic extracts were analyzed using GC-EI-MS and LC-ESI-MS. To isolate unknown compounds, the products were extracted with CHCl3 under ultrasonication and the extracts were evaporated to dryness. After fractionation of the extracts by a liquid-liquid partition method, these unknown compounds were isolated. Structure elucidation of these compounds was performed primarily by nuclear magnetic resonance (NMR) analyses and LC-QTOF-MS. **Results:** Two new designer drugs, MT-45 (1-cyclohexyl-4-(1,2-diphenylethyl) piperazine, synonym: I-C6) and Noopept (ethyl 2-(1-(2-phenylacetyl)pyrrolidine-2-carboxamido)acetate , synonym: GVS-111), were identified in some of chemical and herbal products. A piperazine derivative, MT-45, was reported as an opiate-like analgesic substance. Additionally, a synthetic peptide, Noopept, is sold as a dietary supplement to have a nootropic (cognitive enhancer) activity. Furthermore, we have found that another product contains four major ingredients: two synthetic cannabinoids, A-834735 and QUPIC N-(5-fluoropentyl) analog (synonym: 5-fluoro-PB-22), MT-45 and Noopept. A-834735, which also has been newly detected in illegal products, was known to act as an agonist at both cannabinoid CB1 and CB2 receptors. **Conclusions:** Considering the results, new types of designer drugs rapidly appear and their combinations in illegal products seem to be more diversifying than before.

Keywords: Synthetic cannabinoid, Piperazine derivative, Synthetic peptide, Opiate, Nootropic, Designer drug

OE_{13}
DETERMINATION OF MAM-2201 AND ITS METABOLITES IN A FATAL CASE AND THE BINDING AFFINITIES OF MAM-2201 AT THE CANNABINOID CB1 AND CB2 RECEPTORS

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Introduction: In recent years, herbal products adulterated with synthetic cannabinoids have been widespread in global illegal drug markets. In this study, quantitative analyses for MAM-2201 (synonym: JWH-122 N-(5-fluoropentyl) analog), its oxidative metabolites (including dehalogenated metabolites) and other prescribed medicines were carried out in a fatal case caused by the smoking of the herbal products. Moreover, the binding affinities of MAM-2201 at the human cannabinoid CB1 and CB2 receptors were measured to investigate the possible pharmacological activity. **Materials and Methods:** 1) Samples: Blood, serum, hair and urine samples were obtained from the man who died after smoking herbal products. 2) Target compounds for quantitative analyses: The synthetic cannabinoids (mainly MAM-2201) detected in the herbal products, three putative oxidative metabolites of MAM-2201 (including the dehalogenated metabolites) and three psychotropic medicines prescribed before death (sulpiride, brotizolam and ethyl loflazepate) were focused upon in this study. 3) Analytical method: Chromatographic separation of UPLC-MS/MS was performed in a gradient mode (0.1% formic acid - acetonitrile) using an ACQUITY UPLC BEH C18 column. MRM was used in the positive mode of an ESI-MS/MS for the quantitative analyses. For the screening analyses, UPLC-QTOF MS was carried out in a gradient mode (ammonium formate buffer, pH 3 - acetonitrile) using an ACQUITY UPLC HSS C18 column. 4) Sample preparation: Liquid-liquid extraction with t-butyl methyl ether was used for urine samples after enzymatic hydrolysis, and hair samples after washing with methanol, followed by alkali hydrolysis. Blood and serum samples were analyzed after protein precipitation, using a mixture of methanol and acetnitrile. 5) In vitro binding assay: The evaluation of the IC50 values of MAM-2201 was based on the competitive interaction between a labeled ligand ([3H] CP-55,940) and an analyte (MAM-2201) for the human cannabinoid CB1 and CB2 receptors binding sites. These values were compared to those of the structurally-related compound JWH-122. **Results:** MAM-2201 (73.5 - 81.7 mg/g) was mainly detected in the products possibly related to the death. The oxidative dehalogenated metabolite of MAM-2201: JWH-122 N-(5-hydroxypentyl)

metabolite (45.3 and 100.4 ng/mL) was detected in the blood and serum samples with sulpiride (1.1 and 0.9 µg/mL), followed by MAM-2201(4.4 and 5.5 ng/mL), brotizolam (5.0 and 2.5 ng/mL) and other dehalogenated metabolite: JWH-122 N-pentanoic acid (0.2 and 0.6 ng/mL). In the urine sample, JWH-122 N-(5-hydroxypentyl) metabolite (3.4 ng/mL) was detected as the major metabolite and the parent compound MAM-2201 was also detected at 0.6 ng/mL. Suliride (15.5 µg/mL) was detected in the urine without enzymatic hydrolysis although this compound was degraded under the condition of the hydrolysis. In the wash solvent and the alkali-hydrolyzed extract from the hair, MAM-2201 was mainly detected. A large amount of sulpiride was detected in the biological samples because its daily dosage for therapeutic purpose was 150-300 mg. One of the putative specific metabolites of MAM-2201, MAM-2201 N-(4-hydroxypentyl) metabolite was not detected in any samples. Zolpidem, a psychotropic medicine but not prescribed before the death, was also detected in the hair extract as a result of the screening analyses using UPLC-QTOF MS. The CB1 and CB2 receptors binding affinities of MAM-2201 were 9.5 and 1.6 times bigger than those of JWH-122, based on the IC50 values measured in this study. **Conclusions:** JWH-122 was reported to have acute toxicities probably related to the high-affinity and high-efficacy agonist of the CB1 receptor. Therefore, potential serious health damage may be expected after the intake of MAM-2201. Moreover, unexpected effects associated with the intake of MAM-2201 and the psychotropic medicines are also causing concern.

Keywords: Synthetic cannabinoids, MAM-2201, Oxidative metabolites, Cannabinoid CB1/CB2 receptor binding affinity

OE_{14}
FORENSIC PROFILING OF HEROIN SIDE COMPOUNDS BY CAPILLARY ELECTROPHORESIS

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General Abstract: Forensic profiling of drugs of abuse is a powerful method to gain information about geographic origin of samples, production processes or for tracking the raw material. For heroin profiling, forensic scientists aim at understanding the different processing steps from opium up to heroin and its possible cutting agents. Authorities analyze these complex mixtures usually by GC-FID, focusing on a few analytes whose concentration is highly discriminating. However, regarding the number of impurities and their diverse character, the use of several ideally orthogonal separation techniques is of interest for in-depth profiling. Capillary electrophoresis can deal with analytes which are difficult to be analyzed by GC due to their high hydrophilicity and possible charge or thermal instability. Capillary electrophoresis coupled to mass spectrometry has been shown to be well suited for the identification and quantification of the components of complex plant extracts. In this work we present a CE-MS method, which can deal with raw opium and heroin made thereof. Analysis time is for all three types of samples below 20 min. The method is robust against matrix effects and needs only methanolic extraction and dilution as sample preparation step. The method was applied to a model sample set of opium and heroin from different seizures and the raw data from the electropherograms were pretreated with an in-house software. The program is capable to align CE-MS data based on characteristic m/z-values to correct for shifts in the migration time and to automatically pick peaks according to a predefined mass list of analytes of interest. The resulting information is then merged into a peak table for further data handling and statistics. The peak tables were subjected to a principal component analysis. Several types of opium could be differentiated and unique markers for the discrimination were extracted. In summary, a fast and reliable CE-MS method for the screening of opium, heroin and acidic extracts was developed and applied for profiling purposes. The method provides sufficient information on seizures of illicit drugs for discrimination as well as information orthogonal to classical GC approaches.

Keywords: Opium, Heroin, Chemometrics, Alignment

OE_{15}
APPLICATION OF LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR ANALYSIS OF THE WHOLE BLOOD FOR DESOXYPIPRADROL (2-DPMP) AND STRUCTURALLY RELATED DRUGS - DIPHENYLPROLINOL (D2PM), PHENCYCLIDINE (PCP), KETAMINE AND NORKETAMINE

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Introduction: Recently, many substances, that have been known for many years, reappeared in a drug market. In 2011 and 2012 nine seized powders positive for 2-DPMP, 3 cases (capsules) with D2PM and 1 powder containing PCP were analyzed in the Institute of Forensic Research (IFR). In 2012 our laboratory received two cases in which screening analyses for 'legal highs' carried out by LC-MS revealed positive results for desoxypipradrol (2-DPMP). Therefore, it seemed appropriate to develop a method for simultaneous determination and quantification of PCP and its structural derivatives such as ketamine, norketamine, 2-DPMP and D2PM in blood. In first case 20-year-old woman who was hospitalised with suspicion of poisoning with narcotic drugs received from unknown man. The blood samples were taken after two days of the event and submitted to IFR. The second case concerned 20-year-old man who committed suicide. Due to the suspicion that the man took drugs, the autopsy blood was taken for toxicological analysis. **Materials and Methods:** 0.2 mL blood samples were spiked with analytes and ISs (PCP-D5 and amphetamine-D5) and extracted from alkaline medium (200 µL of 0.5 M carbonate buffer, pH 12) with 1 mL of n-butyl chloride and back extracted with 100 µL of 0.025 M HCl. Organic layer was evaporated (40-45°C), remaining in the vials aqueous phase (acidic) and analyzed with 6460 Triple Quad LC/MS (Agilent Technology). The components were separated on Zorbax SB-C18 (50x2.1mm) column using gradient elution of 0.1% (v/v) formic acid in water and acetonitrile. The target compounds were identified and quantified using MRM mode. The following precursor ions and fragment ions for each compound, selected as qualifiers and quantifiers (shown as firs) were monitored:

21%. **Conclusions:** Hence, our routine procedure proved to be a robust screening method for the detection of various drugs in postmortem brain material.
Keywords: Postmortem, Brain, Drugs of abuse, Validation

PP7

A FATAL CASE AFTER SMOKING HERBS CONTAINING A SYNTHETIC CANNABINOID MAM-2201

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Introduction: Synthetic cannabinoids, such as "Spice" and "herbal blends" have widespread globally in drug markets. It has been reported that use of synthetic cannabinoids can cause a variety of psychoactive and physical effects. Although a case report about intoxication of MAM-2201, one of synthetic cannabinoids, has been published recently, a fatal case that MAM-2201 is related to the cause of death has not been reported. We report a case a man who probably died of cardiopulmonary arrest suddenly after smoking of MAM-2201 and forwarded for autopsy. **Case Report:** A 26-year-old man had a history of depression for about 10 years and he had been prescribed 3 kinds of medicine (sulpiride, brotizolam and ethyl loflazepate). He was smoking herbs which contain MAM-2201 mainly after drinking beer in his friend's room. Immediately after smoking the herbs using a metal pipe, he complained that his limbs became numb and he vomited several times. A few minutes later, he fell unconscious. When the rescue team arrived 30 min later, he had cardiopulmonary arrest. Despite resuscitation in hospital, he died 13 hrs after smoking. A forensic autopsy was performed approximately 3 days after death. **Results and Discussion:** The deceased was 176 cm tall and 62 kg in weight. Autopsy findings including histopathological examination did not reveal anything remarkable cause of death except cerebral swelling and sign of cerebral hypoxia pathologically. MAM-2201 and its two putative metabolites (JWH-122 N-5OH, JWH-122 N-COOH), sulpiride, and brotizolam in serum and blood obtained at the hospital were analyzed by LC-MS/MS1). The concentration of MAM-2201 and JWH-122 N-5OH in serum was 5.46 and 100.43 ng/mL, respectively. The serum level of MAM-2201 about 1 hr after smoking was quite lower than the reported intoxication level (49 ng/mL) in plasma2). The concentrations of both sulpiride (0.92 ?g/mL) and brotizolam (4.98 ng/mL) in plasma were therapeutic level. These compounds were also detected and quantified in blood, serum, urine and hair samples obtained at the autopsy. However, brotizolam was detected in neither urine nor hair samples. These results indicate that the deceased was smoking MAM-2201 at detectable level. The alcohol and standard drug screening tests in blood were negative. **Conclusions:** The findings of autopsy did not show any major abnormality except symptom of cerebral hypoxia leaving the cause of death ambiguous. Toxicological analyses revealed concentration of MAM-2201 in serum was much lower than the intoxication level of MAM-2201 according to the previously published paper2). Therefore, it is unlikely that the death was caused solely by MAM-2201. However, his condition became worse suddenly after smoking of MAM-2201 and he had cardiopulmonary arrest finally within 30 min after smoking, indicating that MAM-2201 was indeed a trigger to the fatal consequences. Taken together, it is plausible that an unknown interaction between MAM-2201 and psychotropic medicine might worsen the adverse effect of this drug.
Keywords: Synthetic cannabinoids, MAM-2201, Autopsy, Psychotropic medicine

PP8

VALIDATED ANALYTICAL METHOD FOR THE DETERMINATION OF ALDICARB IN HUMAN POSTMORTEM WHOLE BLOOD BY HPLC-DAD

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Introduction: The carbamate aldicarb (Temik ® 150), a reversible inhibitor of the enzyme cholinesterase, is classified as highly toxic substance. In Brazil, in 2011, it was estimated that 60% of 8,000 cases of human poisoning by granulated pesticides, were related to aldicarb. In fact, the misuse and illegal trade of this product, become a public health problem in some Brazilian states, leading to even cancellation of Temik 150 ® registration in Brazil in October 2012. Annually the Forensic Toxicology Coordination analyzes cases of fatal poisoning by aldicarb, thus it has the need to establish a method of quantitative analysis for this substance. This paper presents a quantitative analytical method, validated for aldicarb determination in human postmortem whole blood samples, using the technique of high efficiency liquid chromatography with diode array detector, HPLC-DAD, and concentration data obtained for samples of actual cases analyzed. **Materials and Methods:** Aldicarb standard solution, at different concentration levels, was spiked to human postmortem negative whole blood aliquots contained in 2 mL conical polypropylene tubes. The final sample volume was 500 µL. Liquid-liquid extractions were proceeded according to the following sequence: addition of 100 µL of 0.1 M HCl, and gentle manual homogenization for 10 seconds, addition of 400 µL of Dichloromethane, manual and smooth homogenization for 1 minute. After centrifugation at 13,000 rpm for 5 minutes, 200 µL of organic phase was collected and transferred to a clean 2 mL conical polypropylene tube. The extracts were reduced to dryness under nitrogen flow and reconstituted with 10 µL of carbaryl standard solution 215.57 µg/mL as internal standard and 190 µL of HPLC mobile phase consisting of acetonitrile: phosphate buffer pH 2.63 (37:63 v/v). A 50 µL aliquot was injected for analysis. The chromatographic separation was performed on a Merck Lichrosphere 100RP-8 (250 mm x 4 mm x 5 µm) column with isocratic run at ambient temperature and mobile phase flow rate of 1.0 mL/min. The detection was obtained on a diode array detector (DAD) at the wavelength range: 195 - 380 nm. **Results:** The calibration curve was linear from 0.72 µg/mL (lower limit of quantification) to 10.19 µg/mL, with linear correlation coefficients (R) higher than 0.99. The method

いわゆる「脱法ハーブ」吸入による急死の一剖検例

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【概要】20 歳代男性。約 10 年前からうつ病で 3 種の薬物を服用中。某日同僚と飲酒中いわゆる脱法ハーブの煙を吸入したところ、間もなく意識を消失した。病院到着時は心肺停止状態で、一旦は心拍再開したが約 13 時間後に死亡した。

【解剖所見】身長 176cm、体重 62.2kg。背面に死斑が強く発現、硬直は全関節で強い。脳は 1870g と腫脹し低酸素脳症の状態であるが、その他諸臓器に肉眼的、組織学的異常は認められない。

【薬物検査】植物片及び諸試料中の薬物分析は国立医薬品食品衛生研究所に依頼した。GC-MS、LC-MSで分析の結果、植物片3種類からいずれも合成カンナビノイドであるMAM-2201が検出された。LC-MS/MSで分析の結果、病院収容時の試料からMAM-2201が4.42ng/mL（全血）、5.46ng/mL（血清）、MAM-2201の推定代謝物であるJWH-122 N-(5-hydroxypentyl)metaboliteが45.33ng/mL（全血）、100.43ng/mL（血清）、同じく推定代謝物のJWH-122 N-pentanoic acid metaboliteが0.21ng/mL（全血）、0.58ng/mL（血清）が検出された。剖検時に採取した血液、尿からも同様の3物質が検出された。また病院収容時及び剖検時の全血、血清から処方薬であるSulpirideとBrotizolamが、剖検時の尿中からSulpirideが検出された。

【結語】本屍には低酸素脳症以外に有意な所見はなかった。血液、尿から合成カンナビノイドと処方薬の成分が検出されたが、脱法ハーブ吸入に関する剖検例報告は少なく致死濃度も定かではない。今後、各機関からの事例の積み重ねによる集約的検討が必要と考えられた。

28amL-071

薬毒物試験法　II − 6.　大麻試験法 6・2　カンナビノイド受容体作動薬 2. アミノアルキルインドール類（ナフトイルインドール類）

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合田　幸広[1], 袴塚　高志[1]（[1]国立衛研）

【目的】急増する違法ドラッグ製品による健康被害に対応するため，2013 年 3 月より，カンナビノイド受容体作動薬のうち，ナフトイルインドール構造に特定の置換基を有する化合物群が，薬事法に規定の指定薬物として包括的に規制された．本発表では，ナフトイルインドール構造を有する化合物のうち，既に麻薬として規制されている JWH-018, JWH-122, AM2201, MAM-2201 及びそれらと類似の構造を有する指定薬物 6 化合物（計 10 化合物）について，試験法を提案する．
【方法】試料: 上記麻薬 4 化合物及び構造類似指定薬物 6 化合物 JWH-019, JWH-210, JWH-213, JWH-122 *N*-(4-pentenyl) analog, EAM-2201, 4-MeO-AM2201 を用いた．TLC：シリカゲルプレートに試料溶液を点着後，5 種の展開溶媒で展開し，マルキス試液等で検出した．GC-MS：微極性キャピラリーカラムを用いて昇温条件下，EI 法により分析を行った．LC-PDA-MS：10 mM ギ酸アンモニウム水溶液（pH 3.5）−アセトニトリル系移動相によるグラジエント条件下，ODS カラムを用いて分離し，UV 及び ESI（ポジティブ）法により検出した．
【結果】TLC：展開溶媒にヘキサン−酢酸エチルを用いた系で最も良好に分離し，マルキス試液もしくは 10%硫酸噴霧後加熱による呈色で良好に検出可能であった．呈色はいずれも黄褐色-黄色を示した．GC-MS：いずれも分子イオンがベースピークとして検出され，他にカルボニル由来のヒドロキシラジカルが脱離した[M^+-17]等，構造由来のフラグメントイオンが多数検出された．LC-PDA-MS：各化合物は ODS カラムにより分離可能であった．PDA 検出器において 312-316 nm 付近に極大吸収を示し，また ESI ポジティブモードにおいて，プロトン付加分子イオンが明瞭に検出された．

28pmL-117

法規制植物のLAMPを用いた簡易検出法の検討

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【目的】違法ドラッグ市場において，植物片に合成カンナビノイド等を添加した，いわゆる"脱法ハーブ"と呼ばれる製品が広く流通している．我々はこれら違法ドラッグ製品の乱用防止対策を目的として，継続的に化学的，分子生物学的手法を用いた流通実態調査を行っており，これまでに，少数ではあるが製品中の植物片からアサやサルビア・ディビノラム由来のDNA断片を検出している．そこで本研究ではLoop-Mediated Isothermal Amplification (LAMP) を用いた簡便な法規制植物の検出法を検討したので報告する．

【方法】法規制植物として，アサ，ケシおよびサルビア・ディビノラムを用い，各植物種の特異的塩基配列を有すると考えられる *Tetrahydrocannabinolic/Cannbidiolic acid synthases* のコンセンサス配列, *Thebaine 6-O-demethylase* の第二エクソンおよび *5S rRNA* non transcribed spacer 全長の各領域から，それぞれLAMPプライマーセットを設計した．LoopampDNA増幅試薬キットを用い63°Cの等温条件下で60分の反応を行い，Hydroxynaphthol Blue (HNB) を検出試薬として目視判定による検出能力の検討を行った．分析DNAサンプルは，本法規制植物種とその近縁種および国立衛研で入手した"脱法ハーブ"製品を用いた．

【結果及び考察】上記条件において検討を行ったところ，各プライマーセットはそれぞれ高い特異性を示した．また，各分析サンプルの通常のPCRによる結果とLAMPによる判定結果は完全に一致した．HNBによる目視判定は蛍光検出器を必要とせず，HNBを反応前に反応溶液中に添加できる点は，反応産物によるコンタミの危険性を回避できる優れた検出法であり，違法ドラッグ市場に流通する膨大な"脱法ハーブ"のスクリーニング法の有効な手段であると示唆された．

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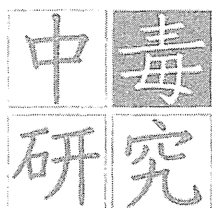

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75 簡易薬物スクリーニングキットを用いた合成カンナビノイドの識別法の検討

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【目的】近年、いわゆる「脱法ハーブ」や「リキッドアロマ」などと呼ばれる違法ドラッグ製品に起因すると考えられる健康被害や交通事故等が問題となっている。また、既報から、特に「脱法ハーブ」には“合成カンナビノイド”が含有している場合が多いと考えられる。一方、救急医療機関等では、簡易的に薬物を識別する際に、市販の簡易薬物スクリーニングキットが使用されている。既存のキットとしてTriage DOAなどがあるが、いずれも“合成カンナビノイド”は対象となっていない。従って現状では、救急の現場において、“合成カンナビノイド”を簡易的に識別する術がない。そこで、本研究では、主にnaphthoylindole型合成カンナビノイド（JWH-018、JWH-073等）を検出対象薬物とした市販の簡易スクリーニングキット“K2/Spice Test”を用いて、法規制された合成カンナビノイドを中心として検討を行うこととした。本キットは、本来尿試料中の対象薬物の代謝物の検出を目的として用いるが、本研究では、化合物本体について本キットを用いた検出が可能であるかを検討した。

【方法】12種類の異なる骨格の合成カンナビノイド38化合物（麻薬：7化合物、指定薬物：25化合物、未規制：6化合物、うちnaphthoylindole型：13化合物）及び大麻の主活性成分Δ^9-THC（麻薬）の計39化合物を使用した。また、違法ドラッグ製品については、植物系3製品（違法ドラッグ成分未検出：2製品、JWH-018含有：1製品）及び「ダミアナ」1製品を用いた。薬物簡易スクリーニングキットは、Drug check® K2/Spice Test（Express Diagnostics社）を用いた。本キットは、JWH-018及びJWH-073の主代謝物に特異的なモノクローナル抗体を用いたイムノアッセイキットである。各化合物の試料溶液は、10% MeOH / 2% emurphor® EL-620 / 88%Water（v/v）の溶液とした。

【結果・考察】検討の結果、naphthoylindole型10化合物、その他2化合物の計12化合物が陽性であった（検出濃度250 μg/mL以上）。このうち、現在麻薬として規制されているnaphthoylindole型5化合物（JWH-018、JWH-073、JWH-122、AM-2201、MAM-2201）は全て検出可能であった。また、例外はあるものの、概ねnaphthoylindole型合成カンナビノイドに特化した検出が可能であると考えられた。また、植物系違法ドラッグ（脱法ハーブ）製品抽出溶液中の薬物も検出が可能であった。従って、本薬物スクリーニングキットは、主にnaphthoylindole型合成カンナビノイドの簡易検出法として有用であると考えられ、今後、救急医療機関などにおける活用の可能性が示された。

合成カンナビノイドのCB1受容体に対する結合様式解析に関する研究

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【目的】乱用が懸念される合成カンナビノイド含有製品（違法ドラッグ）を迅速に規制するためには，その有害性を把握し評価することが重要である．そこで，合成カンナビノイドとカンナビノイド受容体（CB1受容体）の結合様式を化学計算により予測し，さらに化合物の代謝構造を予測することで，違法薬物の規制におけるコンピュータシミュレーションの有用性について検証することを目的とした．

【方法・結果】ロドプシン(PDB:1F88)のX線構造をテンプレートとして，MOE(Molecular Operating Environment；CCG社）を用いてCB1受容体のホモロジーモデル構築（力場；AMBER12:EHT）を行った．さらにSite Finderを用いてモデル構築したCB1受容体のリガンド結合領域を検出し，CB1リガンド（JWH-018およびΔ^{8}-THC）とのドッキングシミュレーション（力場；MMFF94X）を行うことで，CB1受容体に対するリガンドの結合様式の解析を行ったので，それらの詳細について発表する．

forensic science

ODA 10

SCREENING AND QUANTITATIVE ANALYSES OF NEWLY-EMERGED PSYCHOACTIVE SUBSTANCES IN 4 FATAL CASES USING UPLC-MS/MS

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Aims: For rapid and sensitive screening analyses, an UPLC-MS/MS analytical method for 130 newly-emerged psychoactive substances (NPS) in human biological samples was developed. Moreover, this method was applied to examine for the NPS in serum and urine samples obtained from 4 fatal cases, and the quantitative analyses were also carried out for the substances detected in the samples. **Methods:** The optimum conditions for the ionizations of 130 NPS (65 synthetic cannabinoids, 37 cathinone derivatives and 28 other substances) in human control biological samples were evaluated as well as their retention times and detection limits. Chromatographic separation was performed in a gradient mode (0.1% formic acid and acetonitrile) using a C18 column. Human serum and urine samples were obtained from 4 fatal cases caused by the consumption of liquid or herbal products adulterated with NPS. The serum samples were analyzed after protein precipitation, and the urine samples were extracted with t-butyl methyl ether after enzymatic hydrolysis. **Results:** The screening method based on the MRM analyses for the 130 substances were established within 40 min and their detection limits ranged from 0.05 to 0.1 ng/mL. As a result of the screening analyses for the 4 fatal cases, 16 cathinone derivatives, AH-7921, 5F-PB-22, 5F-AB-PINACA and their oxidative metabolites were detected in the serum and urine samples. Especially, 12 cathinone derivatives and AH-7921 were simultaneously detected in one fatal case. By the quantitative analyses of the serum sample from this case, MDPPP (587.4±30.1 ng/mL), AH-7921 (235.5±10.0 ng/mL), MPHP (114.2±5.9 ng/mL), ⊠-PHPP (92.9±4.7 ng/mL) and 4F-⊠-PVP (30.1±0.6 ng/mL) were mainly detected and 8 other cathinone derivatives were less than 5.0 ng/mL. **Conclusions:** In the last 3 years, the number, types and combinations of NPS in illegal products have been diversifying in Japan, and unexpected effects associated with the intake of mixtures of various NPS are causing concern.

Keywords: new psychoactive substances, fatal cases, biological samples, LC-MS/MS