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Anti-HIV benzylisoquinoline alkaloids and flavonoids from the leaves of *Nelumbo nucifera*, and structure–activity correlations with related alkaloids

Yoshiki Kashiwada,[a,*] Akihiro Aoshima,[a] Yasumasa Ikeshiro,[a] Yuh-Pan Chen,[b] Hiroshi Furukawa,[c] Masataka Itoigawa,[d] Toshihiro Fujioka,[e] Kunihide Mihashi,[e] L. Mark Cosentino,[f] Susan L. Morris-Natschke[g] and Kuo-Hsiung Lee[g,*]

[a]*Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata 950-2081, Japan*
[b]*Brion Research Institute of Taiwan, Taipei, Taiwan 100, Republic of China*
[c]*Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya 468, Japan*
[d]*Tokai Gakuen University, Miyoshi, Aichi 470-0207, Japan*
[e]*Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan*
[f]*BBI-Biotech Research Laboratories, Perry Parkway, Gaithersburg, MD 2087, USA*
[g]*Natural Products Laboratory, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA*

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Abstract—(+)-1(*R*)-Coclaurine (**1**) and (−)-1(*S*)-norcoclaurine (**3**), together with quercetin 3-*O*-β-D-glucuronide (**4**), were isolated from the leaves of *Nelumbo nucifera* (Nymphaceae), and identified as anti-HIV principles. Compounds **1** and **3** demonstrated potent anti-HIV activity with EC_{50} values of 0.8 and <0.8 μg/mL, respectively, and therapeutic index (TI) values of >125 and >25, respectively. Compound **4** was less potent (EC_{50} 2 μg/mL). In a structure–activity relationship study, other benzylisoquinoline, aporphine, and bisbenzylisoquinoline alkaloids, including liensinine (**14**), negferine (**15**), and isoliensinine (**16**), which were previously isolated from the leaves and embryo of *Nelumbo nucifera*, were evaluated for anti-HIV activity. Compounds **14–16** showed potent anti-HIV activities with EC_{50} values of <0.8 μg/mL and TI values of >9.9, >8.6, and >6.5, respectively. Nuciferine (**12**), an aporphine alkaloid, had an EC_{50} value of 0.8 μg/mL and TI of 36. In addition, synthetic coclaurine analogs were also evaluated. Compounds **1**, **3**, **12**, and **14–16** can serve as new leads for further development of anti-AIDS agents.

1. Introduction

Nelumbo nucifera Gaertn. (Nymphaceae) is a perennial aquatic crop grown and consumed throughout Asia. All parts of *N. nucifera* have been used for various medicinal purposes in oriental medicine. In particular, the leaves are known for diuretic and astringent properties, and are used to treat fever, sweating, and strangury and as a styptic.[1] During our continuing search for plant-derived anti-HIV agents from natural products, the 95% EtOH extract of the leaves of *N. nucifera* was found to display significant anti-HIV activity (EC_{50} <20 μg/mL, TI > 5). Bioassay-guided fractionation and repeated chromatography of this extract has led to the isolation and identification of (+)-1(*R*)-coclaurine (**1**)[2] and (−)-1(*S*)-norcoclaurine (**3**),[3] together with quercetin 3-*O*-β-D-glucuronide (**4**),[4] as anti-HIV principles. We report herein the anti-HIV activity of **1**, **3**, and **4**, as well as related alkaloids isolated from *N. nucifera* and synthetic coclaurine analogs.

2. Results and discussion

The leaves of *N. nucifera* were extracted successively with *n*-hexane, $CHCl_3$ and 95% EtOH, and anti-HIV activity was found in the 95% EtOH extract (EC_{50} < 20 μg/mL). The 95% EtOH extract was further partitioned between *n*-BuOH and water yielding an

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* Corresponding authors. Tel.: +81 25 269 3140; fax: +81 25 268 1230; e-mail: kasiwada@niigata-pharm.ac.jp

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anti-HIV active *n*-BuOH-soluble fraction. Subsequent bioassay-guided fractionation and repeated chromatography of this fraction led to the isolation of two benzylisoquinoline alkaloids (**1** and **3**) and a flavonoid glycoside (**4**) as anti-HIV principles. Compounds **1** and **3** were identified as (+)-1(*R*)-coclaurine (**1**)[2] and (−)-1(*S*)-norcoclaurine (**3**),[3] respectively, by comparison of their physical and spectral data with those reported in the literature. Although the enantiomer of **3**, (+)-1(*R*)–norcoclaurine, was previously isolated from the embryo of *N. nucifera*[5] this is the first example of the isolation of (−)-1(*S*)-norcoclaurine (**3**) from the leaves of *N. nucifera*. The major component of the leaves of *N. nucifera* was identified as the flavonoid quercetin 3-*O*-β-D-glucuronide (**4**) by comparison of its physical and spectral data with those reported in the literature.[4] In addition, (−)-1(*R*)-*N*-methylcoclaurine (**2**),[6] quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside (**5**),[7] rutin (**6**),[8] isoquecitrin (**7**),[8] and hyperin (**8**)[8] were also isolated and identified from the 95% EtOH extract (Fig. 1).

Alkaloids **1** and **3** demonstrated potent anti-HIV activity with EC_{50} values of 0.8 and <0.8 μg/mL, respectively, and therapeutic index (TI) values of >125 and >25, respectively. Although (−)-1(*R*)-*N*-methyl coclaurine (**2**) showed comparable anti-HIV activity (EC_{50} < 0.8 μg/mL), *N*-methylation led to increased cytotoxicity (IC_{50} 1.45 μg/mL for **2** compared with >100 μg/mL for **1**). Quercetin glucuronide **4** showed moderate anti-HIV activity (EC_{50} 2 μg/mL) and low cytotoxicity (IC_{50} >100 μg/mL), resulting in a TI value of >50. Flavonoid **5** also showed weak anti-HIV activity with an EC_{50} value of 4 μg/mL and a TI of >25, while the remaining quercetin glycosides (**6**–**8**) showed no anti-HIV activity. As quercetin itself also did not show anti-HIV activity (IC_{50} 7.24 μg/mL, EC_{50} no suppression), the glycosyl moiety bound to the C-3 hydroxyl group might play an important role in the anti-HIV activity.

Based on the data of compounds **1**–**3**, benzylisoquinoline-type [*d,l*-armepavine (**9**),[9] and lotusine (**10**)],[4] aporphine-type [roemerine (**11**),[10] nuciferine (**12**),[10] and nornuciferine (**13**)],[10] and bisbenzylisoquinoline-type [liensinine (**14**),[11] neferine (**15**),[12] and isoliensinine (**16**)][11] alkaloids, which were previously isolated from the leaves and embryo of *Nelumbo nucifera*, were evaluated for anti-HIV activity in a structure–activity relationship study. Lotusine (**10**), a quaternary amine alkaloid, showed low cytotoxicity (IC_{50} > 100 μg/mL), but also was not a potent anti-HIV agent. Similar to **2**, the *N*-methyl derivative *d,l*-armepavine (**9**) showed good anti-HIV activity (EC_{50} < 0.8 μg/mL), but was also cytotoxic (IC_{50} 1.77 μg/mL). Bisbenzylisoquinoline-type alkaloids **14**–**16** showed potent anti-HIV activities with EC_{50} values of <0.8 μg/mL, and they were less cytotoxic than **2** or **9**, although they had the same partial structures. In addition, the aporphine-type alkaloids **11**–**13**, which are *N*-methyl derivatives similar to **2** and **9**, were also less cytotoxic as compared with **2** and **9**. Nuciferine (**12**) and nornuciferine (**13**) demonstrated potent anti-HIV activity with EC_{50} values of 0.8 and <0.8 μg/mL, respectively, and TI values of 36.3 and >44, respectively. Roemerine (**11**) showed comparable anti-HIV activity

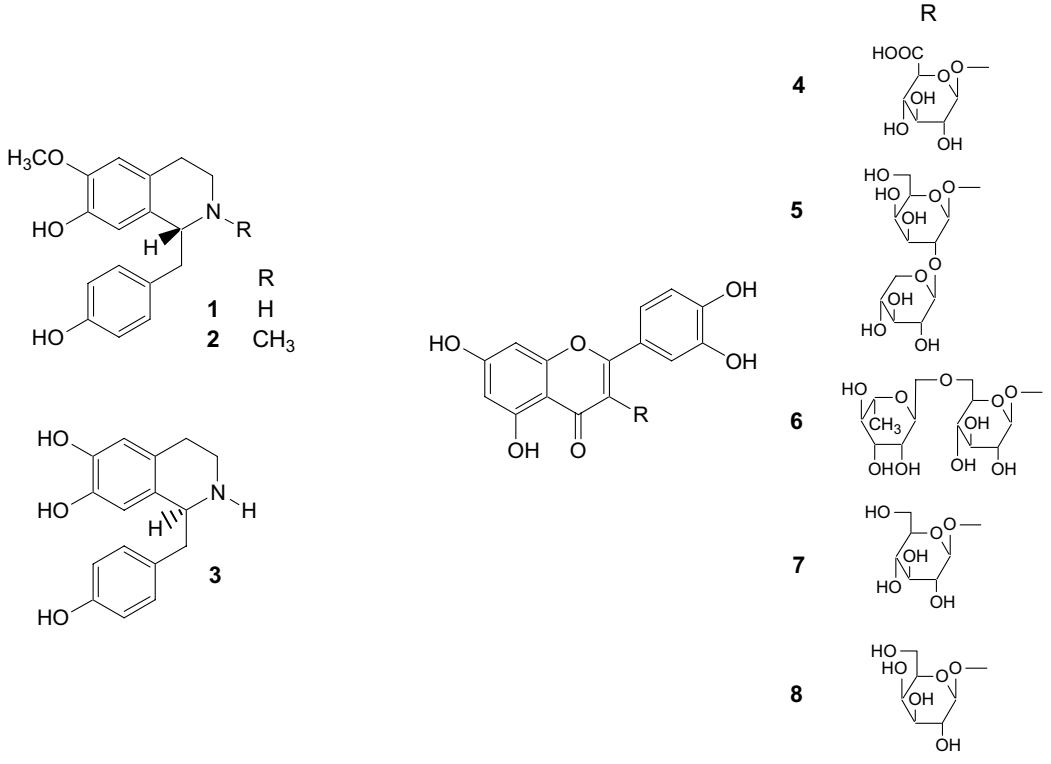

Figure 1. Structures of alkaloids (**1**–**3**) and flavonoids (**4**–**8**) isolated from the leaves of *N. nucifera*.

(EC_{50} 0.84 μg/mL), but had a lower TI value (TI 7.8) than **12** and **13**. These results suggested that *N*-methyl benzylisoquinoline-type alkaloids (such as **9**) were more cytotoxic, whereas oxidative coupling at C-8 (bisbenzylisoquinoline-type, such as **12**) or dimerization at C-2′ (aporphine-type, such as **14**) yielded less cytotoxic compounds.

To investigate the effect of various substitutions in the benzyl ring, racemic coclaurine analogs were prepared according to a previously reported procedure[13] and evaluated for anti-HIV activity. Compounds with additional OMe groups (**17**, **18**) and with halogen rather than OMe (**19**–**21**) were inactive. Compounds **26** and **27**, which are *N*-benzyl-7-benzyl derivatives of the 4′-fluoro and -chloro analogs **20** and **21**, showed weak anti-HIV activity with EC_{50} values of 5.3 and 3.2 μg/mL, but also had low TI values (Fig. 2).

3. Experimental

3.1. General experimental procedures

Melting points were measured on a Yanako micro melting point apparatus, and are uncorrected. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. Mass spectra were determined on a JEOL HX-110 spectrometer. ^1H and ^{13}C NMR spectra were measured on JEOL JNM-A-400 and JNM-FX200 spectrometers with TMS as an internal standard.

3.2. Isolation of alkaloids and flavonoids from the leaves of *Nelumbo nucifera*

The dried leaves of *N. nucifera* (3.0 kg) were extracted successively with *n*-hexane, $CHCl_3$, and 95% EtOH, three times at reflux for 2 h. The combined extracts were

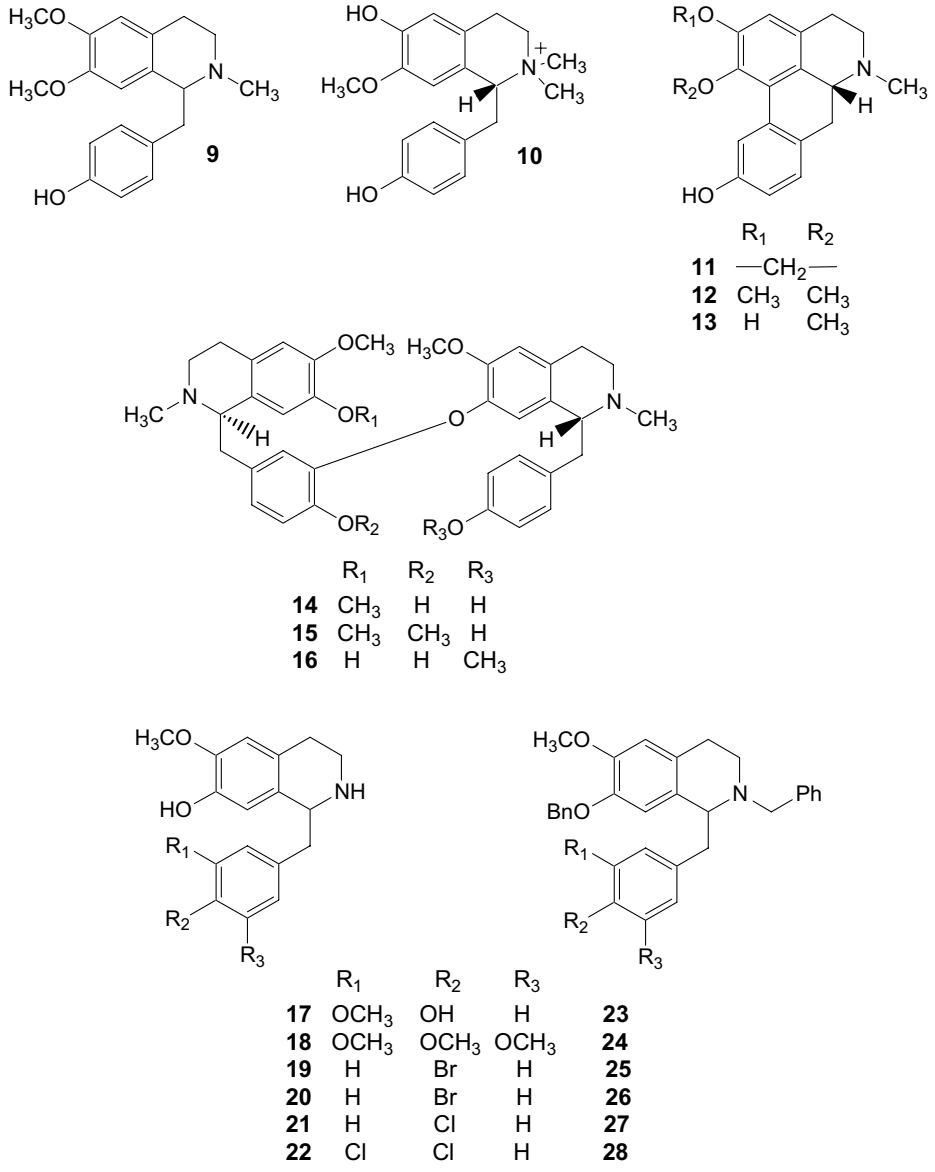

Figure 2. Structures for coclaurine related alkaloids of *N. nucifera* (**9**–**16**) and synthesized benzylisoquinoline alkaloids (**17**–**28**).

concentrated under reduced pressure to give hexane (15 g), $CHCl_3$ (90 g), and 95% EtOH extracts (460 g). The 95% EtOH extract was further partitioned between *n*-BuOH and water yielding *n*-BuOH-soluble fraction, which was subsequently subjected to Diaion HP20 chromatography. Elution with H_2O containing increasing amounts of MeOH gave five fractions, Frs. I–V. Fraction II consisted mainly of compound **4**, which was crystallized from 0.5 M HCl to yield 1.0 g of pure compound. The mother liquor was chromatographed over MCI-gel CHP20P [H_2O–MeOH (1:0→0:1)] to give compounds **1** (175 mg) and **3** (87 mg). Fraction III was further fractionated by MCI-gel CHP20P chromatography with H_2O–MeOH (1:0→0:1) to give fractions III-1–III-3. Subsequent fractionation of Fr. III-1 by Sephadex LH-20 (50% MeOH–80% MeOH) chromatography gave two fractions (frs. III-1-1 and III-1-2). Fraction III-1-1 was chromatographed over Toyopearl HW40F [H_2O–MeOH (1:0→0:1)], and then separated by preparative scale HPLC on Nova Pak HRC18 [MeOH–2% AcOH (2:3)] to furnish compounds **5** (1.45 g) and **6** (60 mg). After crystallization of compound **4** (2.0 g) from fraction III-1-2 by treating with 0.5 M HCl, the mother liquor was further chromatographed on MCI-gel CHP20P [H_2O–MeOH (1:0→0:1)] to give compound **1** (100 mg). Column chromatography of Fraction III-2 over Sephadex LH-20 [H_2O–MeOH (1:0→0:1)], followed by crystallization gave compound **4** (3.2 g). The mother liquor was subsequently chromatographed on MCI-gel CHP20P [H_2O–MeOH (1:0→0:1)] to furnish compounds **3** (420 mg) and **2** (28 mg). Column chromatography of Fraction IV over MCI-gel CHP20P [H_2O–MeOH (7:3→0:1)] and subsequent preparative scale HPLC on Nova Pak HRC18 [MeOH–2% AcOH (2:3)] afforded compounds **7** (330 mg) and **8** (280 mg). All compounds were identified by comparison with literature physical and spectral data. Complete data are given only for **3**, (+)-1(*R*)–norcoclaurine, isolated first herein from this plants source as the *R* enantiomer.

3.2.1. (+)-1(*R*)-Coclaurine (1). Colorless needles; mp 222–224 °C; $[\alpha]_D$ +4.8 (*c* 0.48, MeOH).

3.2.2. (−)-1(*R*)-*N*-Methylcoclaurine (2). Colorless plates; mp 135–137 °C; $[\alpha]_D^{16}$ −115 (*c* 0.6, MeOH).

3.2.3. (−)-1(*S*)-Norcoclaurine (3). Colorless prisms; mp 239–242 °C (decomp.); $[\alpha]_D^{16}$ −39.6 (*c* 0.63, MeOH); ^{1}H NMR (acetone–d_6+D_2O, 400 MHz) δ 2.93, 3.06 (each 1H, dt, J = 6, 17.5 Hz, H_2–4), 3.18 (1H, dd, J = 8, 14.5 Hz, H-7′), 3.39 (1H, dd, J = 6, 14.5 Hz, H-7′), 3.40, 3.56 (each 1H, dt, J = 6, 13 Hz, H_2–3), 4.70 (1H, dd, J = 6, 8 Hz, H-1), 6.70, 6.72 (each 1H, s, H-5, 8), 6.83, 7.21 (each 2H, d,J = 8.5 Hz, H-2′, 3′); ^{13}C NMR (acetone–d_6+D_2O, 100 MHz) δ 39.6 (C-7′), 40.4 (C-3), 57.0 (C-1), 114.0 (C-5), 115.9 (C-8), 116.4 (C-3′,5′), 123.4 (C-5a), 123.7 (C-8a). 126.7 (C-1′), 131.5 (C-2′,6′), 144.8 (C-6), 145.7 (C-7), 157.3 (C-4′).

3.2.4. Quercetin 3-*O*-β-D-glucuronide (miquelianin) (4). Pale yellow needles; mp 190–192 °C; $[\alpha]_D^{16}$ −24.2 (*c* 0.66, MeOH).

3.2.5. Quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside (5). Yellow amorphous powder; $[\alpha]_D^{16}$ −101.5 [*c* 0.67, MeOH–H_2O (1:1)].

3.2.6. Rutin (6). Pale yellow needles, mp 193–194 °C (decomp.), $[\alpha]_D^{16}$ +12.3 (*c* 0.7, MeOH).

3.2.7. Isoquecitrin (7). Pale yellow needles, mp 175–177 °C, $[\alpha]_D^{16}$ −134.6 [*c* 0.52, MeOH–H_2O (1:1)].

3.2.8. Hyperin (8). Pale yellow needles, mp 221–222 °C, $[\alpha]_D^{16}$ −392.9 [*c* 0.28, MeOH–H_2O (1:1)].

3.3. General procedure for preparation of coclaurine analogs

The racemic coclaurine analogs were prepared according to the procedure reported previously.[13] Thus, a solution of an appropriate substituted phenylacetic acid (0.6–1.2 mmol) and thionyl chloride (2.8–6.9 mmol) in dry benzene (10 mL) was refluxed for 2–4 h. After removing solvent in vacuo, the residue was dissolved in CH_2Cl_2 (8 mL) and treated with *N*-benzyl-β-(3-methoxy-4-benzyloxy)-phenylamine (0.4–0.8 mmol) in the presence of triethylamine (3 mL) for 1–2 h with stirring. The reaction mixture was diluted with $CHCl_3$ and washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography to give an amide (60–95% yield). A mixture of amide (0.26–0.73 mmol) and phosphorus oxychloride (2.7–8.7 mmol) in dry toluene (10 mL) was refluxed for 1–5 h. The reaction mixture was concentrated to give an oily residue, which was washed with petroleum ether. A solution of the residue in MeOH (10 mL) was treated with $NaBH_4$ (2.6–8.5 mmol) for 1 h with stirring. After addition of acetone (1 mL), the reaction mixture was diluted with 5% NH_4OH (30 mL), and then extracted with $CHCl_3$ three times. The combined $CHCl_3$ extract was washed with H_2O, dried over Na_2SO_4, and concentrated under reduced pressure. The product was purified by silica gel chromatography (hexane–EtOAc or benzene–EtOAc) to give the *N*-benzyl coclaurine analog benzyl ether (yield 75–95%), which was deprotected by hydrogenolysis. A mixture of *N*-benzyl coclaurine analog benzyl ether (0.2–0.52 mmol), Pd–C (23–130 mg), and trifluoroacetic acid (0.3 mL) in EtOH (6 mL) was stirred under a hydrogen atmosphere overnight. After removal of the catalyst by filtration, the filtrate was concentrated to a syrup. The product was crystallized from acetone solution (55–96% yield).

3.3.1. 1-(3′-Methoxy-4′-hydroxybenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (17). White powder, mp 183–185 °C; ^{1}H NMR (400 MHz, CD_3OD): 2.93–3.08 (3H, m, H_2-4 and H-3), 3.25 (1H, dd, J = 8.5, 14.5 Hz, H-7′), 3.36 (1H, dd, J = 5.5, 14.5 Hz, H-7′), 3.46 (1H, m, H-3′), 3.82, 3.84 (each 3H, s, OCH_3), 4.63 (1H, dd, J = 5.5, 8.5 Hz, H-1), 6.67 (1H, s, H-5), 6.77 (1H, s, H-8), 6.74 (1H, dd, J = 1.7, 7.8 Hz, H-6′), 6.80 (1H, d, J = 7.8 Hz, H-5′), 6.83 (1H, d, J = 1.7 Hz, H-2′); FABMS *m*/*z* 316 $(M+H)^+$; HR-FABMS (positive) *m*/*z* 316.1547 (calcd for $C_{18}H_{22}O_4N$, 316.1549).

3.3.2. 1-(3′,4′,5′-Trimethoxy)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (18). Colorless needles, mp 144–147 °C; ^{1}H NMR (200 MHz, CD_3OD): 2.91–3.18 (3H, m, H_2-4 and H-3), 3.26 (1H, m, H-3′), 3.32–3.48 (2H,m, H_2-7), 3.75, 3.84 (each 3H, s, OCH_3), 3.81 (6H, s, OCH_3), 4.70 (1H, t, J = 7.0 Hz, H-1), 6.61 (2H, s, H-2′,6′), 6.69 (1H, s, H-5), 6.78 (1H, s, H-8); FABMS m/z 360 $(M+H)^+$; HR-FABMS (positive) m/z 360.1813 (calcd for $C_{20}H_{26}O_5N$, 360.1811).

3.3.3. 1-(4′-Bromobenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (19). Colorless needles, mp 215–217 °C; ^{1}H NMR (200 MHz, CD_3OD): 2.99–3.15 (2H, m, H_2-4), 3.08 (1H, dd, J = 8.5, 14.5 Hz, H-7′), 3.22 (1H, dd, J = 5.5, 14.5 Hz, H-7′), 3.43–3.66 (2H, m, H_2-3), 3.85 (3H, s, OCH_3), 4.71 (1H, dd, J = 5.5, 8.5 Hz, H-1), 6.62 (1H, s, H-5), 6.79 (1H, s, H-8), 7.38 (2H, d, J = 8.5 Hz, H-3′,5′), 7.39 (2H, d, J = 8.5 Hz, H-2′,6′); FABMS m/z 348 $(M+H)^+$, 346; HR-FABMS (positive) m/z 348.0596 (calcd for $C_{17}H_{19}O_2NBr$, 348.0600).

3.3.4. 1-(4′-Fluorobenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (20). White powder, mp 197–199 °C; ^{1}H NMR (200 MHz, CD_3OD): 3.02–3.20 (4H, m, H_2-4 and 7′), 3.30–3.66 (2H, m, H_2-3), 3.85 (3H, s, OCH_3), 4.66 (1H, t, J = 7.5 Hz, H-1), 6.58 (1H, s, H-5), 6.79 (1H, s, H-8), 7.11 (2H, t, J = 8.5 Hz, H-3′,5′), 7.33 (2H, dd, J = 5.5, 8.5 Hz, H-2′,6′); FABMS m/z 288 $(M+H)^+$; HR-FABMS (positive) m/z 288.1398 (calcd for $C_{17}H_{19}O_2NF$, 288.1400).

3.3.5. 1-(4′-Chlorobenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (21). Colorless granules, mp 206–208 °C; ^{1}H NMR (200 MHz, CD_3OD): 3.03–3.15 (4H, m, H_2-4 and 7′), 3.37–3.56 (2H, m, H_2-3), 3.84 (3H, s, OCH_3), 4.70 (1H, t, J = 7.5 Hz, H-1), 6.61 (1H, s, H-5), 6.78 (1H, s, H-8), 7.36 (4H, br s, aromatic H); FABMS m/z 304 $(M+H)^+$; HR-FABMS (positive) m/z 304.1107 (calcd for $C_{17}H_{18}O_2NCl$, 304.1104).

3.3.6. 1-(3′,4′-Dichlorobenzyl)-6-methoxy-7-hydroxy-1,2,3,4-tetrahydroisoquinoline (22). Colorless needles, mp 189–191 °C; ^{1}H NMR (200 MHz, CD_3OD): 3.03–3.15 (2H, m, H_2-4), 3.29–3.323.30 (2H, m, H_2-7′), 3.43–3.66 (2H, m, H_2-3), 3.85 (3H, s, OCH_3), 4.71 (1H, dd, J = 5.5, 8.5 Hz, H-1), 6.60 (1H, s, H-5), 6.80 (1H, s, H-8), 7.28 (1H, dd, J = 2, 8 Hz, H-2′), 7.37 (1H, d, J = 8 Hz, H-5′), 7.55 (1H, d, J = 2 Hz, H-2′); FABMS m/z 338 $(M+H)^+$; HR-FABMS (positive) m/z 338.0708 (calcd for $C_{17}H_{18}O_2NCl_2$, 338.0714).

3.3.7. *N*-Benzyl-1-(4′-fluorobenzyl)-6-methoxy-7-benzyloxy-1,2,3,4-tetrahydroisoquinoline (26). A white amorphous powder, ^{1}H NMR (200 MHz, $CDCl_3$): 2.4–3.4 (6H in total, m, H2–3,4,7′), 3.68(1H, t, J = 7 Hz, H-1), 3.74 (2H, s, *N*-benzyl CH_2), 3.86 (3H, s, OCH_3), 4.91 (2H, s, benzyl CH_2), 6.20 (1H, s, H-5), 6.61 (1H, s, H-8), 6.91 (1H, dd, J = 3, 8.5 Hz, H-2′,6′), 7.21 (2H, t, J = 8.5 Hz, H-3′,5′), 7.16–7.36 (10H in total, m, aromatic H); FABMS m/z 468 $(M+H)^+$.

3.3.8. *N*-Benzyl-1-(4′-chlorobenzyl)-6-methoxy-7-benzyloxy-1,2,3,4-tetrahydroisoquinoline (27). A white amorphous powder, ^{1}H NMR (200 MHz, $CDCl_3$): 2.4–3.4 (4H in total, m, H_2-3,4), 2.72, 2.90 (1H, dd, J = 6.5, 14 Hz, H_2-7′), 3.68 (1H, t, J = 6.5 Hz, H-1), 3.73 (2H, s, *N*-benzyl CH_2), 3.86 (3H, s, OCH_3), 4.91 (2H, s, benzyl CH_2), 6.19 (1H, s, H-5), 6.61 (1H, s, H-8), 6.91 (2H, d, J = 8.5 Hz, H-3′,5′), 7.24(1H, d, J = 8.5 Hz, H-2′,6′), 7.15–7.40 (10H in total, m, aromatic H); FABMS m/z 484 $(M+H)^+$.

3.4. Anti-HIV assay

The T cell line, H9, was maintained in continuous culture with complete medium (RPMI 1640 with 10% fetal calf serum [FCS] supplemented with L-glutamine) at 5% CO_2 and 37 °C. Aliquots of this cell line were only used in experiments when in log-phase of growth. Test samples were first dissolved in dimethyl sulfoxide (DMSO). The following are the final drug concentrations routinely used for screening: 100, 20, 4, and 0.8 μg/mL. For active agents, additional dilutions are prepared for subsequent testing so that an accurate EC_{50} values (see definition below) could be achieved. As the test samples were being prepared, an aliquot of H9 cells was infected with HIV-1 (IIIB isolate), while another aliquot was mock-infected with complete medium. The mock-infected sample was used for toxicity determinations (IC_{50}, see definition below). The stock virus used for these studies typically had a $TCID_{50}$ value of 10^4 infectious units (IU)/ml. The appropriate amount of virus for multiplicity of infection (m.o.i.) between 0.1 and 0.01 IU/cell was added to the first aliquot of cells. The other aliquot of cells received

Table 1. Anti-HIV activities for benzylisoquinoline and aporphine alkaloids and flavonoids from *Nelumbo nucifera* and synthetic coclaurine analogs[e]

Compound	IC_{50} (μg/mL)[a]	EC_{50} (μg/mL)[b,c]	TI[d]
1	>100	0.8	>125
2	1.45	<0.8	>1.8
3	20	<0.8	>25
4	>100	2.0	>50
5	>100	4.0	>25
6	>100	35.8	>2.8
9	1.77	<0.8	>2.2
10	>100	20.7	>4.8
11	7.8	0.84	9.3
12	29.0	0.8	36.3
13	35.2	< 0.8	>44
14	7.97	<0.8	>9.9
15	6.87	<0.8	>8.6
16	5.17	<0.8	>6.5
26	7.5	5.3	1.4
27	11.9	3.2	3.7
AZT	1871	0.045 ± 0.056[d]	41,667

[a] The agent concentration that inhibited H9 cell growth by 50%.
[b] The agent concentration that inhibited viral replication in H9 cell by 50%.
[c] In vitro therapeutic index (TI) ratio: IC_{50}/EC_{50}.
[d] This EC_{50} value represents the mean and standard deviation of 65 experimentally determined EC_{50} values for AZT.[14]
[e] Compounds **7**, **8**, **17**–**25**, and **28** showed no suppression.

only culture medium and was then incubated under identical conditions to the HIV-infected cells. After a 4 h incubation at 37 °C and 5% CO_2, both cell populations were washed three times with fresh medium and then added to the appropriate wells of a 24 well-plate containing various concentrations of the test drug or culture medium (positive infected control/negative-control drug). In addition, AZT was also assayed during each experiment as a positive-control drug. The plates were incubated at 37 °C and 5% CO_2 for 4 days. Cell-free supernatants were collected on day 4 and tested by an in-house p24 antigen ELISA assay. P24 antigen is a core protein of HIV and, therefore, is an indirect measure of virus present in the supernatants. Toxicity was determined by performing cell counts by a coulter counter on the mock-infected cells, which had either received culture medium (no toxicity) or test sample or AZT. If a test sample had suppressive capability and was not toxic, its effects are reported in the following terms: IC_{50}, the concentration of test sample that was toxic to 50% of the mock-infected cells; EC_{50}, the concentration of the test sample that was able to suppress HIV replication by 50%; and therapeutic index (TI), the ratio of IC_{50} to EC_{50} (Table 1).

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