In Vitro Anti-HIV Activity of Biflavonoids Isolated from *Rhus succedanea* and *Garcinia multiflora*

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Eleven biflavonoids, including amentoflavone (**1**), agathisflavone (**2**), robustaflavone (**3**), hinokiflavone (**4**), volkensiflavone (**5**), morelloflavone (**7**), rhusflavanone (**9**), succedaneaflavanone (**10**), GB-1a (**11**), GB-1a 7″-*O*-*β*-glucoside (**13**), and GB-2a (**14**) isolated from *Rhus succedanea* and *Garcinia multiflora*, as well as their methyl ethers, volkensiflavone hexamethyl ether (**6**), morelloflavone heptamethyl ether (**8**), and GB-1a hexamethyl ether (**12**), were evaluated for their anti-HIV-1 RT activity. The results indicated that compounds **3** and **4** demonstrated similar activity against HIV-1 reverse transcriptase (RT), with IC_{50} values of 65 μM. Compounds **1**, **2**, **7**, **11**, and **14** were moderately active against HIV-1 RT, with IC_{50} values of 119 μM, 100 μM, 116 μM, 236 μM, and 170 μM, respectively. Morelloflavone (**7**) also demonstrated significant antiviral activity against HIV-1 (strain LAV-1) in phytohemagglutinin-stimulated primary human peripheral blood mononuclear cells at an EC_{50} value of 6.9 μM and a selectivity index value of approximately 10. The other biflavonoids were either weakly active, inactive, or not selective against HIV-1 in human lymphocytes.

Since the identification of the human immunodeficiency virus (HIV) as the causative agent of AIDS,[1,2] the search for safe and effective treatments for HIV infection has become a major focus of drug discovery groups around the world. Investigations into the molecular processes of HIV have led to the identification of a number of macromolecular targets for drug design, such as HIV-1 reverse transcriptase (HIV-1 RT), protease and integrase enzymes, and regulatory proteins (e.g., TAT and REV). Other targets include proteins that are involved in virus attachment and fusion. HIV-1 RT is an essential enzyme in the replication of HIV-1, catalyzing the conversion of the single-stranded RNA genome into double-stranded DNA. Furthermore, the RNA-dependent DNA polymerase function of HIV-RT does not have an analog in mammalian cells, and thus is a suitable target for a chemotherapeutic agent.[3]

In the course of our continuing search for anti-HIV agents from natural products, the MeOH extract of twigs and leaves of *Garcinia multiflora* Champ. (Guttiferae) was found to inhibit strongly the polymerase of HIV-1 RT. In previous studies,[4,5] a series of biflavonoids, including GB-1a (**11**), GB-2a (**14**), volkensiflavone (**5**), morelloflavone (**7**), GB-1a-7″-*O*-glucoside (**13**), GB-2a-*O*-glucoside (xanthochymuside), volkensiflavone-7′-*O*-glucoside (spicataside), morelloflavone-7″-*O*-glucoside (fukugiside), and 1,3,6,7-tetrahydroxyxanthone (Chart 1), was isolated from the heartwood of *G. multiflora*. These results prompted us to examine the anti-HIV-1 RT activity of biflavonoids.

The first biflavonoid, a biflavone, was isolated in 1929 by Furukawa from *Ginkgo biloba* L. as a yellow pigment,[6,7] which was later named ginkgetin.[8] Since that time, approximately 100 biflavonoids have been isolated. The biological activities of some biflavonoids have been reported. For instance, ginkgetin possesses peripheral vasodilatation, anti-bradykinin, anti-spasmogenic, and weak anti-HSV-1 activities.[9,10] Garcinikolin stimulates RNA synthesis in rat hepatocyte suspensions.[11] In addition, agathisflavone, kolaviron, GB-1, and GB-2 exhibit hepatoprotective activity.[12,13] Hinokiflavone, kayaflavone, bilobetin, lophirone A, lophiraic acid, and sotetsuflavone inhibit the expression of the Epstein-Barr virus (EBV) genes.[14−17] GB-1 exhibits molluskicidal activity,[18] while daphnodorin A, daphnodorin B, and daphnodorin D possess antimicrobial activity.[19] Hinokiflavone exhibits cytotoxicity against tissue-cultured

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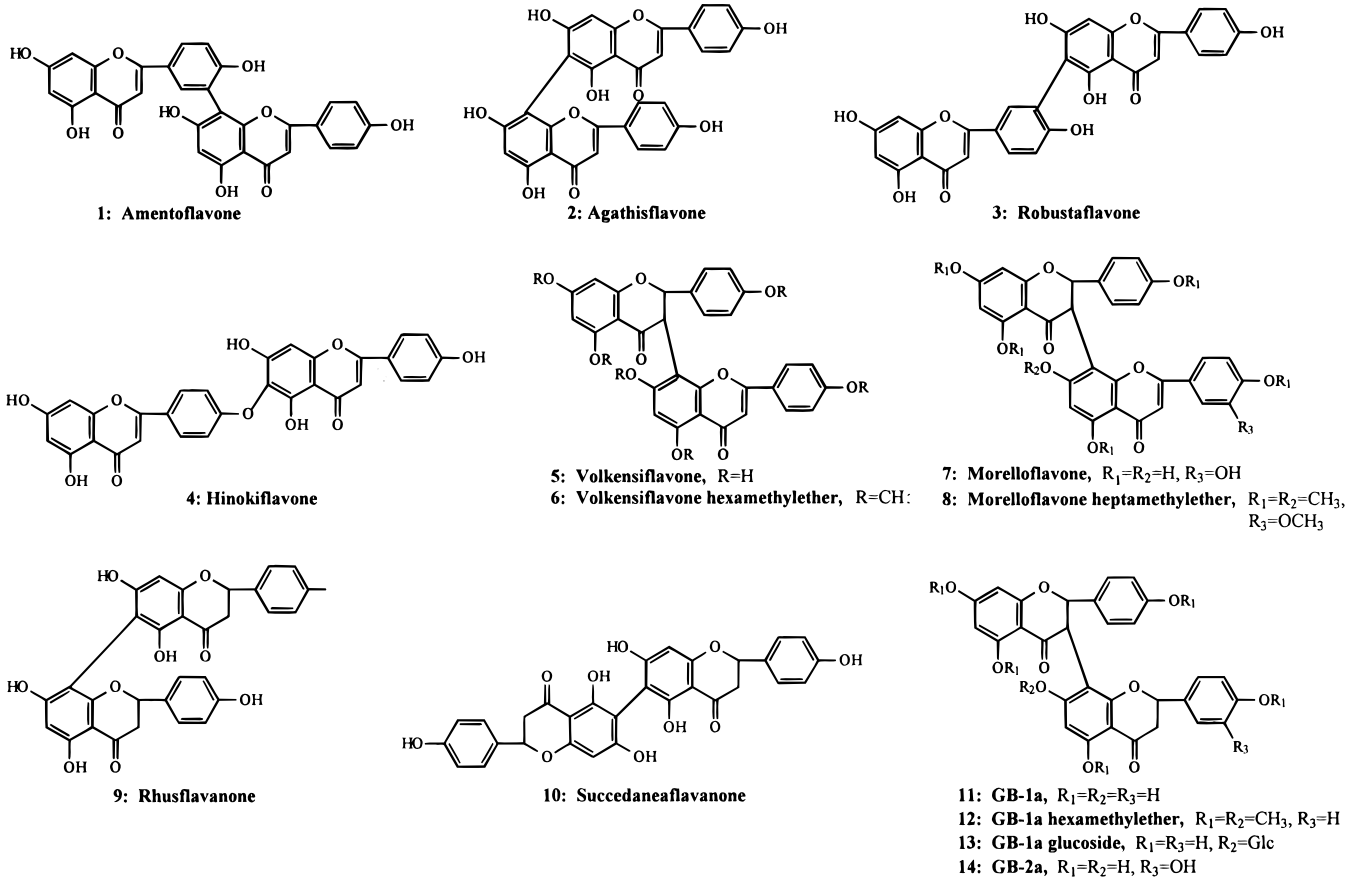
Chart 1



cells of human mouth epidermoid carcinoma (KB)[20] and inhibition of the interleukin-1β-induced expression of tissue factor on human monocytes.[21] Amentoflavone and morelloflavone possess inhibitory effects on lipid peroxidation,[22−24] and kolaviron exhibits hypoglycemic effects.[25]

Previously, we isolated a series of biflavonoids, including amentoflavone (**1**), agathisflavone (**2**), robustaflavone (**3**), hinokiflavone (**4**), rhusflavanone (**9**), and succedaneaflavanone (**10**), from *Rhus succedanea* L. (Anacardiaceae).[26] In this paper, we report the anti-HIV-1 RT activity and efficacy in primary human lymphocytes, acutely infected with HIV-1, of these biflavonoids.

Results and Discussion

The potential of biflavonoids isolated from *R. succedanea*, including amentoflavone (**1**), agathisflavone (**2**), robustaflavone (**3**), hinokiflavone (**4**), rhusflavanone (**9**), and succedaneaflavanone (**10**), and from *G. multiflora*, including volkensiflavone (**5**), morelloflavone (**7**), GB-1a (**11**), GB-1a-7″-*O*-β-glucoside (**13**), GB-2a (**14**), and their methyl ethers, volkensiflavone hexamethyl ether (**6**), morelloflavone heptamethyl ether (**8**), and GB-1a hexamethyl ether (**12**), to inhibit HIV-1 RT and to mediate antiviral activity with HIV-1 acutely infected primary human lymphocytes (PBM) was investigated. The results of these studies are presented in Table 1.

Assays using recombinant HIV-1 RT enzyme (p66/p51 heterodimer) indicated that the biflavones, two apigenin units linked either with C−C or C−O−C bonds, exhibited significant inhibitory activity. Compounds **3** (two apigenins linked through an I-6−II-3′ linkage) and **4** (I-6-O-II-4′ linkage) demonstrated similar activity, with 50% inhibition (IC_{50}) at doses of 65 μM and 62 μM, respectively. The IC_{50} values of **1** (I-8−II-3′ linkage) and **2** (I-6−II-8 linkage) were 119 μM and 100 μM, respectively.

Biflavonoids constructed of flavanone−flavone units through I-3−II-8 linkages were moderately to weakly active, that is, compound **7** (naringenin I-3−II-8 luteolin) demonstrated moderate activity, with an IC_{50} value of 116 μM, while **5** (naringenin I-3−II-8 apigenin) was weakly active. Biflavanones consisting of two naringenin units or naringenin−eriodictol through I-3−II-8 linkages exhibited moderate activity, such as **11** (IC_{50} 236 μM) and **14** (IC_{50} 170 μM). Biflavanones such as **9** and **10**, composed of two naringenin units linked through either I-6−II-8 or I-6−II-6 linkages, were completely inactive.

Structural characteristics were related to activity in our study. Methylation of the hydroxyl groups of the biflavonoids resulted in diminished activity. For instance, compounds **8**, **6**, and **12** were inactive; all exhibited moderate activity prior to alkylation. The fact that GB-1a-7″-glucoside (**13**) demonstrated no activity indicated that the 7″-hydroxyl group was especially important for anti-HIV-1 RT activity.

Six biflavonoids, **1**−**4**, **7**, and **11**, determined to be active in the HIV-1 RT enzyme assay, were tested with human PBM cells infected with HIV-1 (strain LAV-1). These results are presented in Table 1. Although **3** had significant inhibitory activity in the HIV-1 RT enzyme assay, it was inactive in an assay in which PBM cells were infected with HIV-1. However, in the whole cell assay, compound **7** exhibited potent inhibitory activity,

Table 1. Inhibition of HIV-1 RT and Replication in Primary Human Lymphocytes of Biflavonoids

compound	inhibition of HIV-1 RT		anti-HIV-1 activity in PBM cells (EC_{50}(μM)	cytotoxicity in PBM cells IC_{50}(μM)	selectivity index
	% inhibition at 200 μg/mL	IC_{50} (μM)			
G. multiflora (twigs) MeOH extract	99.9	12.5 μg/mL			
G. multiflora (leaves) MeOH extract	99.7	30.7 μg/mL			
apigenin	72.0	443			
naringenin	34.9	weakly active[a]			
amentoflavone (**1**)	97.3	119	94.0	35	<1
agathisflavone (**2**)	99.8	100	33.6	25	<1
robustaflavone (**3**)	91.4	65	>100	77	<1
hinokiflavone (**4**)	89.9	62	4.1	9	2.2
volkensiflavone (**5**)	45.3	weakly active			
volkensiflavone Me_6 (**6**)	0.0	inactive[a]			
morelloflavone (**7**)	99.2	116	6.9	82	12
morelloflavone Me_7 (**8**)	0.0	inactive			
rhusflavanone (**9**)	14.1	inactive			
succedaneaflavanone (**10**)	22.1	inactive			
GB-1a (**11**)	86.0	236	38.0	88	2.3
GB-1a Me_6 (**12**)	0.0	inactive			
GB-1a glucoside (**13**)	1.5	inactive			
GB-2a (**14**)	96.0	170			
AZT (or AZT-TP)			0.009	>100	$>10^4$

[a] Weakly active: 30−50% inhibition at 200 μg/mL; inactive: <30% inhibition at 200 μg/mL.[29]

with an EC_{50} (50% effective dose) value of 6.9 μM, although it only possessed moderate activity in the anti-HIV-1 RT assay (IC_{50} 65 μg/mL; 116 μM). This suggests that the activity of biflavonoids **3** and **7** may be dependent upon different cellular mechanisms or that the cells may be able to selectively concentrate **7**.

Compounds **2**, **4**, and **11** were also active, exhibiting significant activity in inhibiting viral replication in human PBM cells; however, these compounds were highly toxic against uninfected PHA-stimulated human PBM cells. In PBM cells, compound **1** appeared either to lack antiviral potency or to display poor selectivity. From these results, it was concluded that biflavonoid **7**, composed of flavanone (naringenin) and flavone (luteolin) via a I-3−II-8 bond, is the most promising.

Some monoflavonoids have been reported previously to demonstrate anti-HIV activity. Chrysin (5,7-dihydroxyflavone) and acacetin-7-*O*-β-D-galactoside inhibited HIV replication in H9 cells.[27] Baicalein (5,6,7-trihydroxyflavone), tiliroside [kaempferol-3-β-D-(6″-*p*-coumaroyl)-glucoside], quercetin (3,3′,4′,5,7-pentahydroxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), and quercetagetin (3,3′,4′,5,6,7-hexahydroxyflavone) exhibited inhibitory activity against HIV-1 RT, whereas luteolin (3′,4′,5,7-tetrahydroxyflavone) and apigenin (4′,5,7-trihydroxyflavone) demonstrated moderate to slight inhibition, while naringenin (4′,5,7-trihydroxyflavanone) was completely inactive.[28−30] This led to the conclusion that the presence of both the unsaturated double bond between positions 2 and 3 of the flavonoid pyrone ring (e.g., flavone) and the three hydroxyl groups at the 5, 6, and 7 positions (baicalein), or the 3, 3′, and 4′ positions (quercetin), were prerequisites for inhibition of RT.[27,30]

In our study, apigenin exhibited moderate activity, and naringenin showed slight inhibition of HIV-1 RT. Biflavonoids that consisted of two apigenin units (**1**, **2**, **3**, and **4**) demonstrated significant activity. Biflavonoids constructed of flavanone and flavone were moderately active (such as **7**) to weakly active (such as **5**). Biflavonoids linked through I-3−II-8 (**11** and **14**) were moderately active, while biflavanones linked through ring A of two naringenin units (**9** and **10**) were inactive. This structure−activity study demonstrates that hydroxyl groups and at least one flavone unit in the biflavonoids are required for activity. A I-3−II-8 linkage is necessary for biflavanones to exhibit activity, and active compounds become inactive when hydroxyl groups are methylated.

Experimental Section

General Experimental Procedures. Melting points were determined in open glass capillary tubes and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian XL300 NMR spectrometer in $CDCl_3$, DMSO-d_6, or pyridine-d_5, as specified, using TMS as an internal standard. Chemical shifts are expressed in parts per million (δ, ppm). IR spectra were recorded using a Midac FT-IR spectrometer, with samples being prepared as KBr pellets. Mass spectral data were recorded using a Finnegan MAT 90 mass spectrometer. Analytical thin-layer chromatography (TLC) was carried out on precoated plates (Si gel F_{254} from EM Science). Column chromatography was performed with Si gel 60 (70−230 mesh from EM Science). The structures of compounds were confirmed by their TLC profiles as well as their IR, NMR, and MS spectra.

Plant Material. Twigs and leaves of *G. multiflora* were collected in Taichung, Taiwan, in August 1993, by Dr. C.-H. Ou of National Chung-Hsing University, Taichung, Taiwan. Voucher specimens were deposited in the Herbarium of National Chung-Hsing University, Taichung, Taiwan.

Test Compounds. Compounds tested were isolated from the seed kernels of *R. succedanea* obtained from Fukuoka, Japan, and also from the heartwood of *G. multiflora* collected in Taiwan. Compounds **1**,[31] **2**,[32] **3**,[33] **4**,[31] **9**,[26] and **10**[34] were isolated from *R. succedanea*. Compounds **5**, **7**, **11**, **13**, and **14** were isolated from *G. multiflora*.[4] Compounds **6**, **8**, and **12** were prepared from compounds **5**, **7**, and **11**, respectively.[4]

Anti-HIV-1 RT Assay. The HIV-1 RT is a p66/p51 recombinant enzyme obtained using an *Escherichia coli* expression system that contains a genetically engineered plasmid. Synthetic DNA segments were used to introduce initiation and termination codons into the HIV-1 RT coding sequence, which permitted expression

of large quantities of HIV-1 RT in *E. coli*. The enzyme was purified to near homogeneity. The enzyme was shown to be active in RT assays and exhibited inhibitory properties with several known antiretroviral agents (e.g., AZT and suramin) that were indistinguishable from the viral enzyme. The purified recombinant enzyme is sufficiently similar to the viral enzyme that it can be substituted for the latter in drug-screening assays. The recombinant HIV-1 RT preparation used in all experiments had a protein concentration of 0.11 mg/mL and an activity of 238 nmol TTP incorporated per 10 min/mg of protein at 37 °C. Prior to performing an experiment, the enzyme was diluted tenfold with buffer analogous to that used in the assay.

The assay mixture (final volume 100 μL) contained the following: 50 mM Tris−HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethylene glycol-*bis*-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 5 mM dithiothreitol, 0.3 mM glutathione, 2.5 μg/mL bovine serum albumin, 41 μM poly A [Σ_{260} (mM) = 7.8], 9.5 μM oligo $(dT)_{12-18}$ [Σ_{265} (μM) = 5.6], 0.05% Triton X-100, 20 μM TTP, and 0.5 μCi of {^{3}H} TTP. The reaction was initiated by the addition of 10 μL of HIV-1 RT, and the mixture was permitted to incubate at 37 °C for 1 h. Reactions were terminated by the addition of 25 μL of 0.1 M EGTA followed by chilling in ice. Aliquots of each reaction mixture (100 μL) were then spotted uniformly onto circular 2.5-cm DE-81 (Whatman) filters, kept at ambient temperature for 15 min, and washed four times with 5% aqueous $Na_2HPO_4 \cdot 7\ H_2O$. This was followed by two more washings with H_2O. Finally, the filters were thoroughly dried and subjected to scintillation counting in a nonaqueous scintillation fluid.

For testing enzyme inhibition, five serial dilutions of samples in DMSO (10 μL) were added to the reaction mixtures prior to the addition of enzyme (10 μL). The final DMSO concentration in the reaction was 10%. The highest concentration of pure compounds and plant extracts tested was 200 μg/mL. Control assays were performed without the compounds or extracts, but an equivalent volume of DMSO was added. Fargaronine chloride was used as the positive control substance. This compound was isolated from *Fagara xanthoxyloides* Lam. Other positive controls were suramin (IC_{50} 18 μg/mL) and daunomycin (IC_{50} 125 μg/mL). The assay procedure and the concentration of all components were the same as those mentioned above.[29]

Anti-HIV-1 Assay in Primary Human Lymphocyte. Cell culture: Human PBM cells from healthy HIV-1 seronegative and hepatitis B virus seronegative donors were isolated by Ficoll-Hypaque discontinuous gradient centrifugation at 1000 × g for 30 min, washed twice with phosphate-buffered saline (pH 7.2, PBS), and pelleted by centrifugation at 300 × g for 10 min. Before infection, the cells were stimulated by phytohemagglutinin (PHA) at a concentration of 6 μg/mL for 2−3 days in RPMI 1640 medium, supplemented with 15% heat-inactivated fetal calf serum, 1.5 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 4 mM sodium bicarbonate buffer.

Viruses: HIV-1 (strain LAV-1) was obtained from Dr. P. Feorino (Emory University, Atlanta, GA). The virus was propagated in human PBM cells using RPMI 1640 medium, as described previously[35] without PHA or fungizone and supplemented with 26 units/mL of recombinant interleukin-2 (Cetus Corporation, Emeryville, CA) and 7 μg/mL DEAE-dextran (Pharmacia, Uppsala, Sweden). Virus was obtained from cell-free culture supernatant and was titrated and stored in aliquots at −70 °C until use.

Inhibition of virus replication in human PBM cells: Uninfected PHA-stimulated human PBM cells were infected in bulk with a suitable dilution of virus. The mean RT activity of the inocula was about 60 000 dpm RT activity/10^6 cells/10 mL. This represents, by a limiting dilution method in PBM cells, a multiplicity of infection of about 0.01. After 1 h, the cells were uniformly distributed among 25 cm^2 flasks to give a 5-mL suspension containing about 2 × 10^6 cells/mL each. The samples (at twice their final concentrations) in 5 mL of RPMI 1640 medium, supplemented as described above, were added to the cultures. The cultures were maintained in a humidified 5% CO_2−95% air incubator at 37 °C for six days after injection, at which point all cultures were sampled for supernatant RT activity. Previous studies had indicated that maximum RT levels were obtained at that time.

RT activity assay: A volume of supernatant (1 mL) from each culture was clarified of cells at 300 × g for 10 min. Virus particles were pelleted at 12 000 rpm for 2 h using a Jouan refrigerated microcentrifuge (Model MR 1822) and suspended in 100 μL of virus-disrupting buffer (50 mM Tris−HCl, pH 7.8, 800 mM NaCl, 20% glycerol, 0.5 mM phenylmethyl sulfonyl fluoride, and 0.5% Triton X-100).

The RT assays were performed in 96-well microtiter plates, as described by Spira *et al.*[36] The reaction mixture, which contained 50 mM Tris−HCl, pH 7.8, 9 mM $MgCl_2$, 5 mM dithiothreitol, 4.7 μg/mL $(rA)n(dT)_{12-18}$, 140 μM dAPT, and 0.22 μM [^{3}H]TTP (specific activity 78.0 Ci/mmol, equivalent to 17 300 cpm/pmol; NEN Research Products, Boston, MA), was added to each well. The sample (20 μL) was added to the reaction mixture, which was then incubated at 37 °C for 2 h. The reaction was terminated by the addition of 100 μL of 10% trichloroacetic acid (TCA) containing 0.45 mM sodium pyrophosphate. The acid-insoluble nucleic acids that which precipitated were collected on glass filters. The filters were washed with 5% TCA and 70% EtOH, dried, and placed in scintillation vials. A volume of 4 mL of scintillation fluid (Ecolite, ICN, Irvine, CA) was added, and the amount of radioactivity in each sample was determined using a Beckman liquid scintillation analyzer (Model LS 3801). The results were expressed in dpm/mL of original clarified supernatant. The procedures for the anti-HIV-1 assays in PBM cells described above have been published in more detail.[37,38]

Cytotoxicity studies in PBM cells: The compounds were evaluated for their potential toxic effects on uninfected PHA-stimulated human PBM cells. The cells were cultured with and without drug for 24 h, at which time radiolabeled thymidine was added. The assay was performed as described previously.[39] Alternately, cells are counted on day 6 using a hemacytometer and/or Coulter counter as described previously.[38]

Median-Effect Method. EC_{50} and IC_{50} values were obtained by analysis of the data using the median-effect equation.[40] These values were derived from the computer-generated median effect plot of the dose−effect data using a commercially available program.[41]

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