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Amino acid derivatives. Part I. Synthesis, antiviral and antitumor evaluation of new α-amino acid esters bearing coumarin side chain

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A series of amino acid esters bearing coumarin (**3–15**) were synthesized and evaluated, *in vitro*, against HIV-1, and bovine viral diarrhea virus (BVDV). The *in vitro* cytotoxicity of **3**–**10** and **12** was assayed against a panel of tumor cell lines consisting of CD4 human T-cells. Compound **14** showed inhibition of HIV-1 with EC_{50} > 1.6 μg mL^{-1}, meanwhile compound **9** exhibited activity against leukaemia (MT4) with CC_{50} = 24 μmol L^{-1}.

Keywords: amino acids, antitumor activity, antiviral activity, coumarin

Several compounds containing coumarin residue are known to possess useful biological activity (1). Geiparvarin, a naturally occurring product, bearing a coumarin nucleus, has been shown to possess significant inhibitory activity against a variety of cell *Walker* 256 carcinoma sarcoma (2). Warfarin and some bis-hydroxycoumarins have been used as oral anticoagulants (3), β-adrenergic blocking agents (4) and vasorelaxant agents (5). Furumi *et al.* (6) have prepared new furanocoumarin ethers of falcarindol, named japonagelol, as novel antiproliferative agents. On the other hand, a new target for the

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development of anti-HIV and antitumor therapies has been reported by the use, *in vivo* and *in vitro*, of amino acid derived heterocycles. Such compounds are the lysyl amide prodrug of 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (7), amino acid derivatives of paclitaxel (8), cysteine-modifying agents (9) and isoquinoline carboxylic acid derivatives as building blocks for HIV protease inhibitors (10).

The virus family *Flaviviridae* includes the genera *Flavivirus*, *Pestivirus* and *Hepacivirus* (hepatitis C viruses, HCVs). BVDV (Bovine Viral Diarrhea Virus) is one of the classical examples of *Flaviviridae*. Only a few examples have been reported for the treatment of BVDV, such as ribavirin (11) and mycophenolate acid (12). Pharmacological properties of coumarins and amino acid derivatives prompted us to prepare new coumarin bearing amino acid derivatives to study, *in vitro*, their activity against tumor models, along with inhibitory activity for HIV-1 and BVDV.

EXPERIMENTAL

Melting points are uncorrected and were measured on a Büchi melting point apparatus B-545 (BÜCHI Labortechnik AG, Switzerland). Microanalytical data were obtained with a Vario, Elementar apparatus (Shimadzu, Japan). NMR spectra were recorded on

Table I. Physical data of the newly prepared compounds

Compd. No.	Yield (%)	M.p. (°C)	Mol. Formula (M_r)	Found/calcd. (%)		
				C	H	N
3	81	98–99	$C_{14}H_{13}NO_6$ (291.26)	57.60 57.73	4.42 4.40	4.67 4.81
4	76	oil	$C_{18}H_{21}NO_6$ (347.14)	62.02 62.24	6.00 6.09	3.89 4.03
5	85	78–80	$C_{15}H_{15}NO_6$ (305.28)	58.86 59.01	4.82 4.95	4.38 4.59
6	71	oil	$C_{17}H_{19}NO_6$	–	–	–
7	56	86–88	$C_{21}H_{17}NO_6$ (367.35)	65.39 65.13	4.66 4.46	3.81 3.67
8	62	105–106	$C_{23}H_{21}NO_6$ (407.42)	67.54 67.81	5.09 5.20	3.18 3.44
9	66	133–134	$C_{23}H_{20}N_2O_6$ (420.41)	65.50 65.71	4.69 4.79	6.45 6.66
10	73	145–146	$C_{21}H_{19}NO_7$ (397.38)	63.22 63.47	4.69 4.82	3.27 3.52
11	59	oil	$C_{36}H_{39}NO_{17}$	–	–	–
12	62	oil	$C_{34}H_{34}NO_{12}$	–	–	–
13	71	121–124	$C_{14}H_{13}NO_5$ (275.08)	60.75 61.09	4.66 4.76	4.89 5.09
14	65	89–94	$C_{21}H_{19}NO_7$ (397.38)	63.22 63.47	4.69 4.82	3.27 3.52

250 and 600 MHz (^{1}H) and 150.91 MHz (^{13}C) spectrometers (Bruker, Germany) with TMS as internal standard and on δ scale in ppm. Signal assignments for protons were identified by selective proton decoupling or by COSY spectra. Heteronuclear assignments were verified by ^{1}H-^{13}C COSY, or HMQC experiments. Mass spectra were recorded on 70 eV EI and FAB MAT 8200 spectrometers (Finnigana MAT, USA), using nitrobenzyl alcohol (NBOH) or glycerol as matrixes.

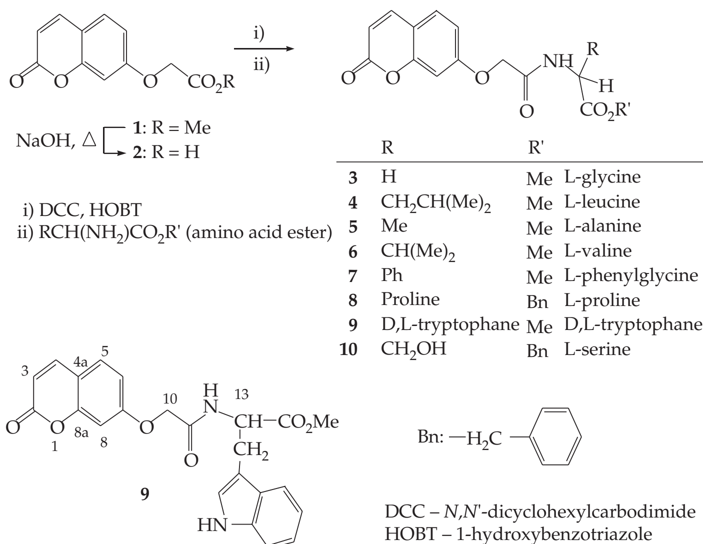
Physicochemical and spectral data for the synthesized compounds are given in Tables I and II. Synthetic routes are presented in Schemes 1–3.

Table II. Spectral data of the newly prepared compounds

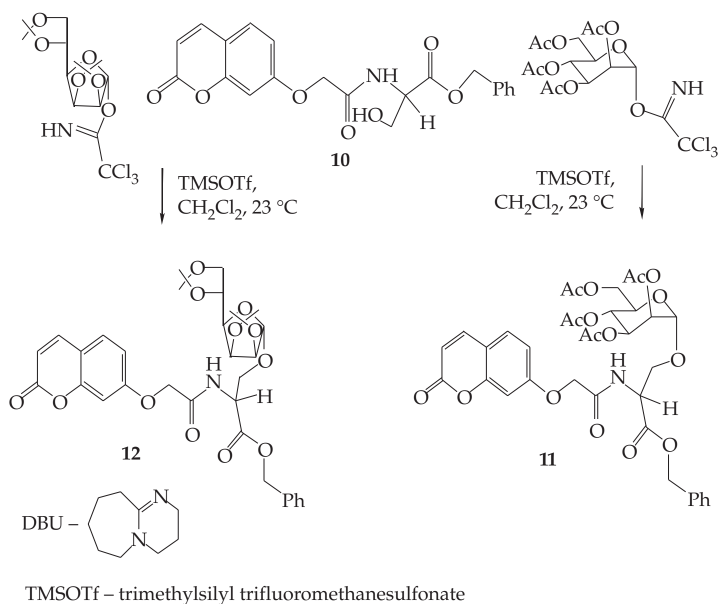
Compd.	Mass (m/z)[a]	^{1}H NMR (δ, ppm)
3	292 $[M+H]^+$	7.62 (d, 1H, *J* = 8.9 Hz, H-4), 7.40 (d, 1H, *J* = 8.9 Hz, H-5), 7.19 (br s, 1H, NH), 6.83 (dd, 1H, *J* = 2.3 Hz, 9.5 Hz, H-6), 6.81 (d, 1H, *J* = 2.3 Hz, H-8), 6.26 (d, 1H, *J* = 9.3 Hz, H-3), 4.56 (s, 2H, CH_2-10), 4.12 (d, 2H, *J* = 2.2 Hz, CH_2-13), 3.73 (s, 3H, OAc), 1.45 (br s, 1H, CH_2-15), 1.09–0.96 (m, 1H, H-16), 0.71, 0.67 (2xs, 6H, Me_2)
4	370 $[M+Na]^+$	7.53 (d, 1H, *J* = 9.6 Hz, H-4), 7.28 (d, 1H, *J* = 8.4 Hz, 2.6 Hz, H-6), 6.68 (d, 1H, *J* = 2.6 Hz, H-8), 6.18 (d, 1H, *J* = 9.5 Hz, H-3), 4.47 (s, 2H, CH_2-10), 4.42 (m, 1H, H-13), 3.57 (s, 3H, OAc), 2.06 (m, 2H, CH_2-15), 1.06 (m, 1H, H-16), 0.78, 0.76 (2s, 6H, 2xMe)
5	306 $[M+H]^+$	7.53 (d, 1H, *J* = 9.3 Hz, H-4), 7.30 (d, 1H, *J* = 8.5 Hz, H-5), 7.05 (d, 1H, *J* = 8.5 Hz, NH), 6.87 (dd, 1H, *J* = 8.6 Hz, 2.5 Hz, H-6), 6.80 (d, 1H, *J* = 2.3 Hz, H-8), 6.17 (d, 1H, *J* = 9.3 Hz, H-3), 4.60 (m, 1H, H-13), 4.56 (s, 2H, CH_2-10), 3.67 (s, 3H, OAc), 1.38, 1.34 (2xd, 3H, *J* = 7.2 Hz, C_{13}-**Me**, two rotameres)
6	356 $[M+Na]^+$	7.53 (d, 1H, *J* = 9.6 Hz, H-4), 7.28 (d, 1H, *J* = 9.6 Hz, H-5), 7.03 (d, 1H, *J* = 8.8 Hz, NH), 6.78 (dd, 1H, *J* = 8.7 Hz, 2.6 Hz, H-6), 6.68 (d, 1H, *J* = 2.6 Hz, H-8), 6.18 (d, 1H, *J* = 9.5 Hz, H-3), 4.47 (s, 2H, CH_2-10), 4.42 (dd, 1H, *J* = 9.5 Hz, 5.1 Hz, H-13), 3.57 (s, 3H, OAc), 2.06 (m, 2H, CH_2-15), 0.78, 0.76 (2s, 6H, 2xMe)
7	390 $[M+Na]^+$	7.67 (d, 1H, *J* = 9.2 Hz, H-4), 7.55 (d, 1H, *J* = 9.5 Hz, H-5), 7.33–7.18 (m, 6H, NH, Ar-H, H-5), 6.80 (dd, 1H, *J* = 8.6 Hz, 2.5 Hz, H-6), 6.72 (d, 1H, *J* = 2.0 Hz, H-8), 6.14 (d, 1H, *J* = 9.5 Hz, H-3), 5.58 (d, 1H, *J* = 7.2 Hz, H-13), 4.49 (s, 2H, CH_2-10), 3.62 (s, 3H, OAc)
8	408 $[M+H]^+$	7.54 (d, 1H, *J* = 9.4 Hz, H-4), 7.29–7.20 (m, 7H, H-5, NH, CH_2**Ph**), 6.82 (dd, 1H, *J* = 8.7 Hz, 2.4 Hz, H-6), 6.72 (d, 1H, *J* = 2.1 Hz, H-8), 6.14 (d, 1H, *J* = 9.5 Hz, H-3), 5.10 (s, 2H,$\mathbf{CH_2}$Ph), 4.69 (s, 2H, CH_2-10), 4.54 (m, 1H, H-16), 3.58 (m, 2H, CH_2-13), 2.29–1.81 (m, 4H, CH_2-14, CH_2-15)
9	398 $[M+H]^+$	9.03 (br s, 1H, NH-indole), 7.48 (m, 2H, H-4, H-5), 7.42–6.51 (m, 5H, NH, indole-5H), 6.54 (m, 2H, H-6, H.8), 6.16 (d, 1H, *J* = 9.4 Hz, H-3), 4.92 (m, 1H, H-13), 4.38 (s, 2H, CH_2-10), 3.67 (s, 3H, OAc), 3.30 (br s, 2H, CH_2-15)
10	398 $[M+H]^+$	7.54 (d, 1H, *J* = 9.4 Hz, H-4), 7.29–7.20 (m, 7H, H-5, NH, CH_2**Ph**), 6.82 (dd, 1H, *J* = 8.7 Hz, 2.4 Hz, H-6), 6.72 (d, 1H, *J* = 2.1 Hz, H-8), 6.14 (d, 1H, *J* = 9.5 Hz, H-3), 5.10 (s, 2H, $\mathbf{CH_2}$Ph), 4.69 (s, 2H, CH_2-10), 4.54 (m, 1H, H-16), 3.58 (m, 2H, CH_2-13), 2.29–1.81 (m, 4H, CH_2-14, CH_2-15)

Table II. continued

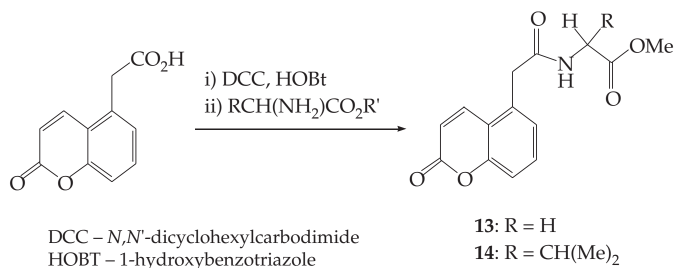
Compd.	Mass (m/z)[a]	^{1}H NMR (δ, ppm)
11	780 $[M+Na]^+$	7.65 (d, 1H, J = 9.6 Hz, H-4), 7.50 (d, 1H, J = 8.1 Hz, NH), 7.43 (d, 1H, J = 8.8 Hz, H-5), 7.38–7.27 (m, 5H, CH$_2$**Ph**), 6.96 (dd, 1H, J = 8.8 Hz, J = 2.0 Hz, H-6), 6.91 (d, 1H, J = 2.0 Hz, H-8), 6.14 (d, 1H, J = 9.5 Hz, H-3), 5.26 (d, 2H, J = 1.2 Hz, **CH$_2$**Ph), 5.24 (m, 2H, H-3', H-4'), 5.15 (br s, 1H, H-2'), 4.93 (m, 1H, H-13), 4.74 (d, 1H, $J_{1',2'}$ = 2.8 Hz, H-1'), 4.62 (s, 2H, CH$_2$-10), 4.19 (dd, 1H, $J_{5',6'a}$ = 5.5 Hz, H-6'a), 4.09 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, $J_{5',6'b}$ = 3.7 Hz, H-6'b), 4.06, 4.00 (d, 2H, J = 4.0 Hz, CH$_2$-17), 3.88 (dt, 1H, $J_{4',5'}$ = 9.5 Hz, H-5'), 2.16, 2.15, 2.07, 1.98 84xs, 12H, 4xOAc)
12	680 $[M+Na]^+$	7.60 (d, 1H, J = 9.6 Hz, H-4), 7.80 (d, 1H, J = 8.0 Hz, NH), 7.37 (d, 1H, J = 8.6 Hz, H-5), 7.35–7.24 (m, 5H, CH$_2$**Ph**), 6.85 (d, 1H, J = 8.5 Hz, H-6), 6.80 (d, 1H, J = 2.4 Hz, H-8), 6.24 (d, 1H, J = 9.5 Hz, H-3), 5.23 (d, 2H, J = 5.7 Hz, H-1'), 5.16 (d, 2H, J = 7.7 Hz, **CH$_2$**Ph), 4.82 (m, 2H, H-2', H-13), 4.67–4.51 (m, 3H, CH$_2$-10, H-4'), 4.74 (d, 1H, $J_{1',2'}$ = 2.8 Hz, H-1'), 4.19 (dd, 1H, $J_{5',6'a}$ = 5.5 Hz, H-6'a), 4.28 (d, 1H, $J_{3',4'}$ = 6.0 Hz, H-3'), 4.27 (dd, 1H, $J_{4',5'}$ = 5.5 Hz, $J_{5',6a'}$ = 7.6 Hz, H-5'), 4.05 (dd, 1H, $J_{5',6'a}$ = 3.7 Hz, H-6'a), 4.01 (m, 1H, H-17a), 3.98 (m, 1H, H-17b), 3.92 (dd, 1H, $J_{6'a,6'b}$ = 12.0 Hz, H-6'b), 1.39, 1.27, 1.25, 1.22 (4xs, 6H, 2xCMe$_2$)
13	680 $[M+H]^+$	7.32 (d, 1H, J = 8.7 Hz, H-5), 7.11 (br s., 1H, NH), 7.19 (dd, 1H, J = 2.4 Hz, 8.7 Hz, H-7), 7.08 (m, 2H, H-6, H-8), 6.17 (s, 1H, H-3), 4.50 (s, 2H, CH$_2$-12), 3.71 (s, 3H, OAc), 2.90 (s, 1H, CH$_2$-9)
14	398 $[M+H]^+$	7.30 (d, 1H, J = 9.4 Hz, H-5), 7.08 (br s., 1H, NH), 7.17 (dd, 1H, J = 2.6 Hz, 9.4 Hz, H-7), 7.07 (m, 2H, H-6, H-8), 6.18 (s, 1H, H-3), 4.45 (s, 2H, CH$_2$-12), 3.60 (s, 3H, OAc), 2.94 (m, 1H, **CH**Me$_2$), 2.89 (s, 2H, CH$_2$-9), 1.00, 0.97 (2s, 6H, CH**Me$_2$**)



Scheme 1



Scheme 2



Scheme 3

*Methyl 2-(2*H*-1-benzopyran-2-one-7-yloxy)acetate (**1**)(13)*

A suspension of 7-hydroxy coumarin (1.0 g, 6.17 mmol) in acetone (20 mL) was refluxed with methyl bromoacetate (1.40 g, 9.15 mmol) and K_2CO_3 (4.69 g, 33.91 mmol) for 12 h. After cooling, the mixture was evaporated to dryness and the residue was partitioned between $CHCl_3$ (50 mL) and water (50 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated to dryness. The residue was recrystallized from acetone to give **1** (1.00 g, 70%, m.p. 150–152 °C; ref. 13: 61%, m.p. 148 °C).

*2-(2H-1-benzopyran-2-one-7-yloxy)acetic acid (**2**)(14)*

A solution of **1** (1.50 g, 6.46 mmol) in EtOH (35 mL) and 5% NaOH (6 mL) was heated under reflux for 2 h. After cooling, the solution was evaporated to dryness and the residue was dissolved in water and acidified with 6 mol L^{-1} HCl. The white precipitate was filtered, dried and crystallized from EtOH to give **2** (1.36 g, 96%), m.p. 218–220 °C, (ref. 14: m.p. 219–220 °C).

*Synthesis of coumarin-bearing amino acid esters (**3–10**)*

General procedure. – To a cold solution of the amino acid ester hydrochloride (10 mmol), at –5 °C, in MeCN (20 mL), 7-coumarin acetic acid (10 mmol), hydroxybenzotriazole (HOBt) (1.35 g, 10 mmol) and *N,N'*-dicyclohexyl-carbodiimide (DCC) (10 mmol) were added successively. The reaction mixture was stirred at 0 °C for 1 h, at 5 °C for 1 h, and at 23 °C for 16 h. Dicyclohexylurea (DCU) was filtered, and the filtrate was evaporated to dryness and the residue was dissolved in ethyl acetate, filtered, washed successively with saturated NaCl solution, 5% $NaHCO_3$ solution, 1.0 mol L^{-1} HCl, followed by washing with saturated NaCl solution and finally with water. The residue was dried (Na_2SO_4), filtered, evaporated to dryness and recrystallized from the appropriate solvent.

*Synthesis of benzyl 2-[2-(2-(2H-benzopyran-2-on-7-yloxy)acetylamino)-3-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranos-1-yloxy)]propanoate (**11**) and benzyl 2-[2-(2-(2H-benzopyran-2-on-7-oxyl)acetylamino]-3-(2,3:4,6-di-O-isopropylidene-α-D-mannofuranos-l-yloxy)]propanoate (**12**)*

General procedure. – A solution of 2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranosyl trichloro--acetimidate or 2,3:4,6-di-*O*-isopropylidene-α-*D*-mannofuranosyl trichloroacetimidate (0.90 mmol) and **10** (358 mg, 0.90 mmol) in dry CH_2Cl_2 (25 mL) was stirred under nitrogen at room temperature for 5 min, followed by the addition of trimethylsilyl trifluoromethanesulphonate (TMSOTf) (19.5 μL, 0.09 mmol). After stirring for 2 h, solid $NaHCO_3$ was added slowly, filtered and the filtrate was washed with water (30 mL), the organic layer was dried (Na_2SO_4), filtered and evaporated to dryness. The residue was purified on SiO_2 column (20 g), using ethyl acetate/petroleum ether as eluent to give **11** and **12**, respectively.

*Synthesis of 4-substituted coumarin with amino acid esters **13** and **14***

General procedure. – The title compounds were prepared by the coupling method applied for preparation of compounds **3–10**, using the amino acid ester hydrochloride (10 mmol), 4-coumarin-acetic acid (10 mmol), hydroxybenzotriazole (HOBt) (1.35 g, 10 mmol) and DCC (10 mmol).

Antitumor screening

Compounds **2–10** and **12** were tested, *in vitro* against a panel of tumor cell lines consisting of CD4 human T-cells containing an integrated human T-leukaemia virus type 1

(HTLV-1), CD4 human acute T-lymphoblastic leukaemia, human splenic B-lymphoblastoid cells, human acute B-lymphoblastic leukaemia, human skin melanoma, human breast adenocarcinoma, human lung squamous carcinoma, human hepatocellular carcinoma, human prostate carcinoma, human foreskin fibroblasts, and human lung fibroblasts. The Microculture Tetrazolium Assay (MTT) method (15) was used for estimation of the *in vitro* tumor-inhibiting activity of the tested compounds. The cell lines of tumor subpanels were incubated within five concentrations (0.01–100 μg mL^{-1}) of each tested compound for 48 h.

RESULTS AND DISCUSSION

The starting material 2-(2*H*-1-benzopyran-2-one-7-yloxy)acetic acid (**2**) was prepared by saponification of the corresponding ester **1** (13) in 96% yield. Compound **2** was prepared previously by direct condensation of 7-hydroxy coumarin with chloroacetic acid, in 61% yield (14).

A suitable coupling method (16) was employed for the formation of peptides by reaction of the carboxylic acid group with acylated amino acid, using 1-hydroxybenzotriazole (HOBt) (17, 18) and *N,N'*-dicyclohexylcarbodiimide (DCC) (19) as coupling reagents. HOBt (**1**) is currently the most frequently used activating agent for the carboxyl group of amino acids. The procedure is fast and suppresses racemization, especially in the presence of DCC (20).

Amides **3**–**10** were prepared by coupling **2** with the appropriate acylated amino acids (L-glycine acetate hydrochloride, L-leucine acetate hydrochloride, L-alanine acetate hydrochloride, L-valine acetate hydrochloride, L-phenylglycine acetate hydrochloride, benzyl-L-proline carboxylate hydrochloride, D,L-tryptophane acetate hydrochloride and benzyl-L-serine carboxylate hydrochloride) in the presence of HOBT and DCC as coupling reagents to give **3**–**10** in 56–85% yield (Scheme 1).

Recently, Schmidt *et al.* (21, 22) reported an efficient anomeric stereocontroled glycosylation method, with high yield of α-anomer, by using *O*-glycosopyranosyl trichloroacetoimidate derivative as donor and alcohol as acceptor precursors in the presence of catalytic amounts of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as Lewis acid. By applying Schmidts procedure, **10** was selected in our present work as alcohol acceptor for coupling with the protected *O*-mannopyranosyl- and *O*-mannofuranosyl-1-trichloroacetimidate derivatives as donor precursors in the synthesis of new glycoside derivatives **11** and **12** in 59% and 62% yield, respectively (Scheme 2). Trichloroacetimidates, characterized by their stabilities at room temperature for long periods of time without any decomposition, were prepared from the reaction of their corresponding 1-hydroxy--protected glycosyl precursors (21, 22) with trichloroacetonitrile in the presence of 1,5-diazobicyclo[5,4,0]undec-5-ene (DBU) as catalyst.

The structures of **3**–**10** were determined by their ^{1}H, ^{13}C NMR and by mass spectra (Tables II and III). The coumarin protons showed a similar pattern. H-3, H-4, H-5 and H-8 protons appeared as doublets in region δ 6.14–6.24 (J = 9.3–9.5 Hz), 7.48–7.67 (J = 8.9–9.6 Hz), 7.28–7.55 (J = 8.4–9.6 Hz) and 6.68–6.81 ppm (J = 2.0–2.6 Hz), respectively. H-6 protons were oriented as a doublet of doublets in the region δ 6.78–6.87 ppm (J =

8.6–9.5 and J = 2.3–2.2.6 Hz), except for **9** which showed multiplets at δ 7.54, attributed to H-3 and H-4 and at δ 6.54 ppm, belonging to H-3 and H-4. The singlets that appeared in the region δ 4.38–4.69 ppm were characterized as CH_2-10 protons. H-13 protons appeared as doublets, a doublet of doublets or multiplets at δ 3.58–4.92 ppm, depending on the functional group adjacent to C-13. The other protons of the amino acid ester were fully analyzed. The ^{13}C NMR spectra of **3–10** contained similar resonance signals of the coumarin carbons ring C-2–C-8a. The chemical shifts between δ_C 161.5 and 161.8 ppm were assigned to the carbonyl group of the benzopyran ring (C-2), while the resonances in the range of δ_C 66.8– 67.4 ppm were assigned to CH_2-10 protons. Compounds **11** and **12** were identified from the ^1H NMR and ^{13}C NMR spectra, which showed almost similar resonance of benzopyran ring atoms as those of **10**. The sugar proton H-1′ of **12** appeared as a doublet at δ 5.23 ppm, while H-2′ was oriented with H-13 as a multiplet at δ 4.82 ppm. H-4′ appeared together with CH_2-10 as a multiplet at δ 4.67–4.51 ppm. The small $J_{1',2'}$ coupling (5.23 Hz) confirmed the α-anomer of glycoside **12**. Assignment of proton resonances of **11** was confirmed by homo decoupling and by COSY spectra. ^1H-^{13}C COSY, DFQ-COSY, HSQC, HMQC and HMBC were used for assignments of the ^{13}C resonances.

The ^{13}C NMR spectrum of **11** showed signals at higher field: δ 170.6–169.6 ppm were attributed to the acetoxy groups of the sugar moiety while resonances at δ 160.6, 167.0, 168.0 ppm were assigned to the carbonyl groups C-2, C-11 and C-14, respectively. C-4a, C-7 and C-8a appeared at δ 113.8, 159.8 and 155.6 ppm, respectively. The gradient selected ^1H,^{13}C-HSQC spectrum in $CDCl_3$ showed couplings between the benzopyran ring H-3–H-8 as well as CH_2-10, H-13 and CH_2-17 at δ 6.14 ppm (d, J = 9.5 Hz), 7.65 ppm (d, J = 9.6 Hz), 7.43 ppm (d, J = 8.8 Hz), 6.96 ppm (d, J = 8.8 Hz), 6.91 ppm (d, J = 2.0 Hz), 4.62 ppm (s), 4.93 ppm (m), 4.00 ppm (d, J = 4.0 Hz) and C-3–C-8, C-10, C-13 and C-17 at δ 114.2, 143.0, 129.2, 112.3, 102.5, 52.4, 52.2, 68.5 ppm, respectively. The small $J_{1',2'}$ coupling (2.8 Hz) is typical of the α-configurated mannopyranoside. The DFQ-COSY spectrum showed a linear correlation between the sugar protons H-1′ (δ 4.74 ppm), H-3′ (δ 4.28 ppm) and the amino acid protons CH_2-10 (δ 4.62 ppm), H-13 (δ 4.93 ppm) and CH_2-17 (δ 4.00 ppm), indicating α-configuration. Additional proof for α-configuration came from the ROESY spectrum, which suggested a large distance between H-1′ and H-5′. The large $J_{4',5'}$ coupling (9.5 Hz) of **11** is indicative of the $^4C_1$ conformation of the pyranose ring. Furthermore, C-17 at δ 68.5 ppm was inferred from the HMBC and HMQC spectra by showing $^3J_{C,H}$ coupling with H-1′ at δ 5.23 ppm.

Further, our work was modified by selecting the 4-coumarin acetic acid (23) as precursor for the synthesis of new derivatives to examine their antiviral activity in comparison to the 7-coumarin analogues **3**–**10**. Compounds **13** and **14** were prepared in 71 and 65% yields from 4-coumarin acetic acid by applying the coupling method used previously in the presence of HOBt and DCC as coupling reagents (Scheme 3). The structures of **13** and **14** were determined from their ^1H NMR and mass spectra.

Antiviral assay

Compounds **3**–**10** were tested for their anti-HIV-1 activity *in vitro*, using III_B strain in human T-lymphocyte (MT-4) cells, based on a Microculture Tetrazolium (MTT) assay and the results are reported in Table IV. Cytotoxicity was also measured on MT-4 cells.

Table III. ^{13}*C NMR data of the newly prepared compounds*

Compd.	^{1}H NMR (*δ*, ppm)
3	172.4 (C-14), 166.7 (C-11), 160.1 (C-2), 159.8 (C-7), 155.1 (8a), 142.8 (C-4), 128.8 (C-5), 113.4 (C-3), 113.2 (C-4a), 112.0 (C-6), 102.0 (C-8), 67.1 (C-10), 51.8 (C-13), 50.0 (C-14), 40.7 (C-15), 24.4 (C-16), 22.3, 21.3 (C_{16}-Me_2)
4	171.8 (C-14), 167.1 (C-11), 160.6 (C-2), 160.1 (C-7), 155.4 (8a), 143.2 (C-4), 129.2 (C-5), 113.7 (C-3), 113.5 (C-4a), 112.4 (C-6), 102.1 (C-8), 67.3 (C-10), 56.8 (C-13), 52.1 (C-14), 30.9 (C-15), 18.8, 17.7 (2xMe)
5	172.8 (C-14), 166.6 (C-11), 160.7 (C-2), 160.1 (C-7), 155.6 (8a), 143.1 (C-4), 129.2 (C-5), 114.0 (C-3), 113.6 (C-4a), 112.3 (C-6), 102.4 (C-8), 67.4 (C-10), 52.6 (C-13), 47.7 (C-14), 18.2 (C_{13}-**Me**)
6	171.8 (C-14), 167.1 (C-11), 160.6 (C-2), 160.1 (C-7), 155.4 (8a), 143.2 (C-4), 129.2 (C-5), 113.7 (C-3), 113.5 (C-4a), 112.4 (C-6), 102.1 (C-8), 67.3 (C-10), 56.8 (C-13), 52.1 (C-14), 30.9 (C-15), 18.8, 17.7 (2xMe)
7	170.8 (C-14), 166.7 (C-11), 160.7 (C-2), 160.1 (C-7), 155.5 (8a), 143.2 (C-4), 135.9 (Ar-C_a), 129.2 (Ar-C_f), 128.9 (Ar-$C_{c,e}$), 128.6 (Ar-C_d), 127.3 (C-5), 113.7 (C-3), 113.5 (C-4a), 112.5 (C-6), 102.2 (C-8), 67.3 (C-10), 56.1 (C-13), 52.8 (C-13), 20.9 (C-14)
8	183.1 (C-17), 171.5 (C-14), 165.8 (C-11), 161.1 (C-2), 160.9 (C-7), 155.5 (8a), 143.4 (C-4), 135.9, 129.0, 128.7, 128.5, 128.3, 128.2 (CH_2*Ar-C*), 127.9 (C-5), 113.4 (C-3), 113.2 (C-4a), 112.6 (C-6), 102.0 (C-8), 67.0 (C-16), 66.8 (C-10), 59.2 (**CH_2**Ph-13), 28.6 (C-15), 24.9 (C-14)
9	170.8 (C-14), 166.7 (C-11), 160.7 (C-2), 160.1 (C-7), 155.5 (8a), 143.2 (C-4), 136.3 (indol-C_i), 129.0 (C-5), 127.4 (indol-C_d), 123.3 (indol-C_b), 122.0 (indol-C_f), 119.6 (indole-C_g), 118.2 (indole-C_e), 113.6 (C-3), 113.5 (C-4a), 112.2 (C-6), 111.7 (indole-C_h), 109.0 (indole-C_c), 102.2 (C-8), 67.2 (C-10), 52.8 (C-13), 52.5 (C-14), 27.3 (CH_2-15)
10	169.8 (C-14), 167.6 (C-11), 161.0 (C-2), 160.0 (C-7), 155.5 (8a), 143.3 (C-4), 129.2 (C-5), 135.1, 128.6, 128.5, 128.1, 128.2 (CH_2**Ar**-C), 127.9 (C-5), 113.9 (C-3), 113.6 (C-4a), 112.4 (C-6), 102.5 (C-8), 67.6 (C-16), 67.4 (C-10), 62.7 (**CH_2**Ph), 54.6 (C-13)
11	170.6, 169.8, 169.7, 169.6 (**CO**Me), 168.8 (C-14), 167.0 (C-11), 160.6 (C-2), 159.8 (C-7), 155.6 (8a), 143.0 (C-4), 129.2 (C-5), 134.8, 128.7, 128.6, 128.3 (CH_2*Ph-C*), 129.2 (C-5), 114.2 (C-3), 113.8 (C-4a), 112.3 (C-6), 102.5 (C-8), 98.2 (C-1′-sugar), 69.3 (C-2′-sugar), 69.2 (C-5′-sugar), 68.6 (C-3′-sugar), 68.5 (C-17), 67.9 [C-16 (**CH_2**Ph)], 67.4 (C-10), 62.3 (C-6′-sugar), 52.2 (C-13), 20.8, 20.7, 20.6, 20.5 (4xCO**Me**)
12	170.1 (C-14), 168.2 (C-11), 160.9 (C-2), 160.2 (C-7), 154.2 (8a), 143.3 (C-4), 135.9 (CH_2**Ph**-C), 129.2 (C-5), 128.9, 128.8, 128.2 (CH_2**Ph**-C), 114.7(C-3), 114.2 (C-4a), 112.6 (C-6), 103.6 (C-8), 105.1 (C-1′-sugar), 84.0 (C-2′-sugar), 80.5 (C-3′-sugar), 78.4 (C-5′-sugar), 76.3 (C-4′-sugar), 72.1 (C-6′-sugar), 68.1 (C-16 **CH_2**Ph), 67.5 (C-10), 63.8 (C-17, CH_2-glycoside), 59.2, 57.1 (Me, acetal groups), 52.7 (C-13)

The gp41 subunit of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein plays an important role in HIV-1 entry and serves as an attractive target for the development of HIV-1 entry inhibitors, a new class of anti-HIV drugs (24). Triggered by gp120 binding to CD4 and a coreceptor, gp41 undergoes a conformation shift from a native prefusogenic state to a fusogenic state, in which the N-terminal heptad repeat (NHR) and C-terminal heptad repeat (CHR) associate to form a six-helix bundle, representing the fusion-active gp41 core. Any compound that disrupts the gp41 sixhelix bun-

dle formation may inhibit the gp41-mediated membrane fusion, thereby blocking HIV-1 entry into target cells (25).

Our target was the distruption of the gp41 sixhelix bundle formation by the newly synthesized amino acids, leading to inhibition of HIV. None of the *in vitro* tested compounds **3**–**10** and **12** were found to inhibit HIV-replication, at EC_{50} lower than the CC_{50} compared to the antiviral agents efavirenz (EFV) (26) and azidothymidine (AZT) (27), whereas compound **11** was not tested against HIV. On the other hand, **13** and **14** were tested against HIV-1 (III_B strain) with EC_{50} > 14 and 1.6 μg mL^{-1}, respectively, and against HIV-2 (ROD strain) with EC_{50} > 19 and 3.2 μg mL^{-1}, respectively, at non-toxic concentrations.

The above data showed no selective anti-HIV activity, except for compound **14**, which showed an encouraging result by inhibiting of HIV with EC_{50} value > 1.6 μg mL^{-1}.

Compounds **3**–**10** and **12** were evaluated against BVDV, and showed no inhibition at non-toxic concentrations, in comparison to the iminosugar derivatives (28), since the minimium inhibitory concentration required to reduce the virus-induced cytopathogenicity by 50% was higher than 100 μg mL^{-1} as shown in Table IV. Compounds **11**, **13** and **14** were not tested against BVDV.

Table IV. In vitro *anti-HIV-1*[a] *and anti-BVDV activity of some coumarin compounds*

Compd.	CC_{50} MT-4 (μg mL^{-1})[b]	EC_{50} HIV-1 (μg mL^{-1})[c]	CC_{50} BVDV (μg mL^{-1})	EC_{50} BVDV (μg mL^{-1})
3	> 100	> 100	> 100	91
4	> 100	> 100	> 100	> 100
5	> 100	> 100	> 100	> 100
6	> 100	> 100	> 100	66
7	> 100	> 100	> 100	100
8	> 100	> 100	> 100	> 100
9	24	> 24	> 100	100
10	> 100	> 100	> 100	> 100
12	49	> 49	> 100	> 100
13	> 14	> 14		
13[d]	> 19	> 19		
14	> 1.6	> 1.6		
14[d]	> 3.2	> 3.2		
EFV	40	0.003		
AZT	63	0.02		

[a] Anti-HIV-1 activity measured with strain III_B.
[b] CC_{50} concentration required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT (Microculture Tetrazolium Assay) method.
[c] EC_{50} concentration required to achieve 50% protection of MT-4 cells from the HIV-1 induced cytopathogenicity, as determined by the MTT method.
[d] Anti-HIV-2 activity measured with strain ROD.
EFV – efavirenz
AZT – azidothymidine

Anticancer assay

The Microculture Tetrazolium Assay (MTT) method (15), which is based on metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was used for preliminary estimation of the *in vitro* tumor-inhibiting activity of the coumarin derivatives **3**–**10** and **12** against a panel of tumor cell lines. The results are summarized in Table V.

Table V. Antitumor activity in most sensitive tumor cell lines

Compd.	Tumor	Cell line	CC_{50} (μmol L^{-1})[a]
9	Leukaemia/	*MT4*[b]	24
	lymphoma	*CCRF-CEM*[c]	36
		WIL-2NS[d]	45
		CCRF-SB[e]	48
	MT-4		24
	MDBK		> 100
	Solid tumor-	*SK-MEL-28*[f]	60
	derived cell lines	*MCF7*[g]	> 100
		SKMES-1[h]	80
		HepG2[i]	> 100
		DU145[j]	90
	Normal-cell lines	CRL 7065[k]	> 100
		MRC-5[l]	> 100
12	Leukaemia/	*MT4*[b]	49
	lymphoma	*CCRF-CEM*[c]	53
		WIL-2NS[d]	68
		CCRF-SB[e]	59
			24
	MT-4		> 100
	MDBK	*SK-MEL-28*[f]	60
	Solid tumor-	*MCF7*[g]	> 100
	derived cell lines	*SKMES-1*[h]	80
		HepG2[i]	> 100
		DU145[j]	90
	Normal-cell lines	CRL 7065[k]	> 100
		MRC-5[l]	> 100

[a] Compound concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication.
[b] CD4 human T-cells containing an integrated HTLV-1.
[c] CD4 human acute T-lymphoblastic leukaemia.
[d] Human splenic B-lymphoblastoid cells.
[e] Human acute B-lymphoblastic leukaemia.
[f] Human skin melanoma.
[g] Human breast adenocarcinoma.
[h] Human lung squamous carcinoma.
[i] Human hepatocellular carcinoma.
[j] Human prostate carcinoma.
[k] Human foreskin fibroblasts.
[l] Human lung fibroblasts.

All the new compounds were inactive against all tumor cell lines (CC_{50} > 100 μmol L^{-1}, except for compounds **9** and **12**, which showed marked activity against leukaemia/lymphoma. Comparing the activity of **9** to the other coumarin bearing amino acid derivatives showed that the inclusion of the indole moiety shifted the threshold of potency from the inactive side toward activity, particularly against the leukaemia cell lines MT4 (CC_{50} = 24 μmol L^{-1}). It is noticed that the substances with aliphatic substituents are inactive whereas the tryptophane derivative **9** shows significant activity. This may be due to the indole residue, which performs more intermolecular interactions.

The introduction of a furanose sugar moiety into the OH group of **12** by glycoside linkage may be responsible for a slight change in the antitumor activity.

CONCLUSIONS

The structure activity relationship suggested that the substituted carbon-coumarin linkage showed higher HIV inhibition than the corresponding analogues having oxygen linkage, and this result should lead us to modify our new target molecules by introducing more potent groups with carbon linkage.

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S A Ž E T A K

Derivati aminokiselina. Dio I. Sinteza, antivirusno i antitumorsko djelovanje novih estera α-aminokiselina s kumarinskim supstituentom

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U radu je opisana sinteza estera aminokiselina s kumarinskim ostatkom **3–15**. Ispitano je antivirusno djelovanje sintetiziranih spojeva na HIV-1 i goveđi virus diareje (BVDV), te *in vitro* citotoksičnost spojeva **3**–**10** i **12** na tumorskim linijama CD4 humanih T-stanica. Spoj **14** pokazao je inhibiciju HIV-1 s EC_{50} > 1,6 μg mL^{-1}, dok je spoj **9** djelotvoran na leukemiju (MT4) s CC_{50} = 24 μmol L^{-1}.

Ključne riječi: aminokiseline, antitumorsko djelovanje, antivirusno djelovanje, kumarin

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