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INHIBITORY EFFECT OF CARNOSOLIC ACID ON HIV-1 PROTEASE IN CELL-FREE ASSAYS

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ABSTRACT.—In order to find new effective HIV protease inhibitors, two diterpenes (carnosolic acid [**1**] and carnosol [**5**]) were isolated from rosemary (*Rosmarinus officinalis* L.), and rosmanol [**2**] and semisynthetic derivatives (7-*O*-methylrosmanol [**3**], 7-*O*-ethylrosmanol [**4**], and 11,12-*O,O*-dimethylcarnosol [**6**]) were prepared. The inhibitory activity of all six compounds against HIV-1 protease was tested. The carnosolic acid [**1**] showed the strongest inhibitory effect (IC_{90}=0.08 μg/ml). The same compound was also assayed against HIV-1 virus replication (IC_{90}=0.32 μg/ml). The cytotoxic TC_{90} on H9 lymphocytes was 0.36 μg/ml, which is very close to the effective antiviral dose. Additionally, the tested compounds did not inhibit cellular aspartic proteases cathepsin D and pepsin at the concentration range up to 10 μg/ml.

All known retroviral genomes code for a virus-specific aspartic protease which is essential for the processing of viral precursor polyproteins into the functional proteins of the mature viral particles and for viral infectivity (1). In the case of HIV-1, etiologic agent of Acquired Immune Deficiency Syndrome (AIDS) (2), the aspartic protease is assembled into an active homodimer of the two identical 99 amino acid polypeptides, each subunit contributing one Asp^{25}-Thr^{26}-Gly^{27} sequence at the active site, which results in molecular architecture analogous to those of eucaryotic aspartic proteases (3,4). Genetic and inhibition studies have shown that the spread of viral infection can be arrested if the protease activity is blocked (5,6). It was reported that upon transfection into mammalian cells, a mutant HIV provirus containing the Asn^{25} mutation within the protease gene produced immature, non-infectious virions containing unprocessed polyproteins (6). Therefore, HIV-1 protease is regarded as a potential target for developing new drugs useful in the treatment of AIDS (7). In contrast to the large number of synthetic HIV-1 protease inhibitors (8), only a few naturally-occurring inhibitors of this enzyme have been discovered so far (5, 9–11).

In earlier investigations of the different diterpenes, many biological effects, such as antioxidative effects, bacteriostatic, cytostatic, and other activities have been reported (12).

In the present work we report the effects of some diterpenes isolated from rosemary and their semisynthetic derivatives on HIV-1 protease activity and HIV virus replication.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^1H and ^{13}C nmr were obtained on a Varian VXR 300 in $CDCl_3$ with TMS as internal standard; mass spectra were recorded with a Finnigan SSQ 70 and VG Superspec; and ir spectra were obtained on Perkin Elmer 1600. Melting points measured on a PHMK 05 are uncorrected. Cc was performed using Merck Si gel 60 (70–230 mesh), and gel permeation chromatography on Pharmacia Sephadex LH20.

PLANT MATERIAL.—The leaves of rosemary, *Rosmarinus officinalis* L. (Labiatae) were collected at the Slovenian coast, air-dried, and ground to a fine powder. A voucher specimen (12/90) has been deposited at the Dept. of Pharmacy, FNT, Ljubljana, Slovenia.

ISOLATION OF CARNOSOLIC ACID [**1**].—Completely dried leaves (500 g) were repeatedly extracted in N_2 atmosphere with *n*-hexane (2 liters). The extract was concentrated to 1/3 of its volume and treated with saturated aqueous $NaHCO_3$ solution. The aqueous phase was immediately washed with *n*-hexane and treated with 2 M HCl to achieve pH 2.0. The acidic solution was subsequently re-extracted with *n*-hexane. The *n*-hexane solution was dried over Na_2SO_4 and concentrated under reduced pressure, until pale yellow crystals appeared. Recrystallization from *n*-hexane yielded pale yellow prisms of compound **1**, mp 189–192°.

PREPARATION OF ROSMANOL [**2**], 7-*O*-METHYLROSMANOL [**3**], AND 7-*O*-ETHYLROSMANOL [**4**].—Rosmanol [**2**] and 7-*O*-methylrosmanol [**3**] were obtained from carnosolic acid dissolved in MeOH-H_2O (5:2) with addition of a catalytic amount of a MeOH solution of NaOH. After 24 h at 25°, the solvent was evaporated and the residue separated by cc on SiO_2 using $CHCl_3$ as eluent. The mixture of the two main compounds was separated on LH-20 column using EtOH as eluent. Compounds **2** and **3** crystallized as colorless prisms, mp 239–240° and mp 188–190°, respectively. Compound **4** (colorless prisms, mp 205–207°) was prepared by an identical procedure except that EtOH was substituted for MeOH.

ISOLATION OF CARNOSOL [**5**] AND PREPARATION OF 11,12-*O,O*-DIMETHYLCARNOSOL [**6**].—Carnosol [**5**] was extracted from powdered rosemary leaves, and 11,12-*O,O*-dimethylcarnosol [**6**] was prepared according to the method described previously (13).

ENZYMES AND SUBSTRATES.—Recombinant HIV-1 protease was isolated from inclusion bodies of *Escherichia coli* BL-21 (DE3) cells containing the expression plasmid pET-11 (14). The cells were grown to mid-log phase and induced with 0.1 mM sopropyl-β-D-thiogalactopyranoside for 60 min at 37° before harvest. Solubilization, refolding, and purification of the recombinant viral protease were as previously described (15).

The cell-free assay of recombinant HIV-1 protease was done using ^{35}S-labeled p55 gag precursor protein, synthesized in a cell-free reticulocyte lysate transcription-translation system (16) containing 1 mCi/ml of ^{35}S-methionine (New England Nuclear Corp.). A portion of the translation mixture was analyzed on an gradient 8–18% polyacrylamide gel, containing SDS.

Cathepsin D (E.C. 3.4.23.5) was isolated from bovine spleen as previously described (17).

Porcine pepsin (E.C. 3.4.23.1) was obtained from Sigma.

HIV-1 PROTEASE ACTIVITY ASSAY.—To the radioactive p55, approximately 1 μg of purified HIV protease was added in the presence or absence of inhibitory compounds and reactions were carried out at 30° for 60 min in phosphate buffered saline (PBS), pH 7.2. Test compounds were dissolved in DMSO at a concentration of 10 mg/ml, stored at −70°, and warmed and diluted appropriately just prior to use. On completion of the assay, samples were adjusted to contain 1% SDS, 1% β-mercaptoethanol, and 7% glycerol and heated to 95° for 1 min. The products were then separated on SDS gels, and the dried gels were used to expose Kodak XAR film. Films were scanned by a Perkin-Elmer recording densitometer for quantitation of inhibitory potency.

HIV YIELD REDUCTION ASSAY.—MT-2, a human T-cell line, was cultured in RPMI medium supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS), L-glutamine, and gentamycin. Human immunodeficiency virus strains, HIV-1 (3b) and HIV-1 (rf) were propagated in H9 cells in RPMI with 5% FCS. Poly-L-lysine (Sigma) coated cell culture plates were prepared according to the method of Harada *et al.* (18). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was obtained from Sigma.

Test compounds were dissolved in DMSO to 5 mg/ml and serially diluted into RPMI medium to ten times the desired final concentration. MT-2 cells (5×10^5/ml in 2.3 ml) were mixed with 0.3 ml of the appropriate test compound solution and allowed to sit for 30 min at room temperature. Virus (5×10^5 pfu/ml) in 0.375 ml was added to the cell and compound mixtures and incubated for 1 h at 36°. The mixtures were centrifuged at 1000 rpm for 10 min, and supernatants containing unattached virus were discarded. The cell pellets were suspended in fresh RPMI containing the appropriate concentrations of test compound and placed in a 36°, 4% CO_2 incubator. Virus was allowed to replicate for 3 days. Cultures were centrifuged for 10 min at 1000 rpm, and the supernatants containing cell-free progeny virus were removed for plaque assay.

The virus titers of the progeny virus produced in presence or absence of test compounds were determined by a plaque assay similar to that previously described (19). Progeny virus suspensions were serially diluted in RPMI, and 1.0 ml of each dilution was added to 9 ml of MT-2 cells in RPMI. Cells and virus were incubated for 3 h at 36° to allow for efficient attachment of the virus to cells. Each virus and cell mixture was aliquoted equally to two wells of a six-well poly-L-lysine-coated culture plate and incubated overnight at 36°, 4% CO_2. Liquid and unattached cells were removed prior to the addition of 1.5 ml of RPMI with 0.75% (w/v) Seaplaque agarose (FMC Corp.) and 5% FCS. Plates were incubated for 3 days, and a second RPMI/agarose overlay was added. After an additional 3 days at 36°, 4% CO_2, a final overlay

of PBS with 0.75% Seaplaque agarose, and 1 mg MTT/ml were added. The plates were incubated overnight at 36°. Clear plaques on a purple background were counted, and the number of plaque forming units of virus was calculated for each sample. The antiviral activity of test compounds was determined by the percent reduction in the virus titer with respect to virus grown in the absence of any inhibitors.

CYTOTOXICITY ASSAY.—Cell viability was determined by the metabolism of the tetrazolium dye, XTT, to a colored formazan product (20). Microtiter plates (96 wells) were seeded with 1×10^5 cells/well and graded concentrations of test compound. Cells were allowed to grow at 36° in the presence of compound for 3 or 5 days depending on the cell type. To each well was added 0.05 ml of 1 mg XTT/ml in PBS containing 0.025 mM phenazine methosulfate. Plates were incubated an additional hour at 36°, and the od_{450} was determined. The od was proportional to the number of viable cells in the well. The concentration of compound which produced a 90% reduction in the number of viable cells was designated the TC_{90}.

CATHEPSIN D AND PEPSIN ACTIVITY ASSAY.—Cathepsin D and pepsin activities were determined by the modified methods of Anson (21). In both cases, the final concentration of the enzyme in reaction mixture was 40 nM.

For cathepsin D assay, a stock solution of each inhibitor in DMSO was diluted with 0.1 M Na acetate buffer pH 3.5 and incubated with enzyme solution in a total volume of 200 µl. After 10 min of incubation, 1.2 ml of 2% hemoglobin in 0.1 M Na acetate buffer was added and incubated for 10 min at 37°. Reaction was stopped by adding 1.4 ml of 0.33 M CCl_3COOH. NaOH (2 ml, 0.5 M) and Folin-Ciolcalteu's phenol reagent (Merck) (1.2 ml) were added to 2 ml of the soluble reaction products obtained by filtration, and after 5 min the absorbance at 750 nm was read.

For pepsin assay, stock solution of each compound was diluted with 0.01 M HCl and incubated at room temperature with enzyme solution in total volume of 600 ml. After 10 min, 1.25 ml of substrate solution (2% Hb, adjusted with 1 M HCl to pH 1.8) was added and incubated at 37° for 10 min. Reaction was terminated by adding 2.6 ml of 0.33 M TCA. The extent of digestion was determined in the filtrate by reading absorbance at 280 nm.

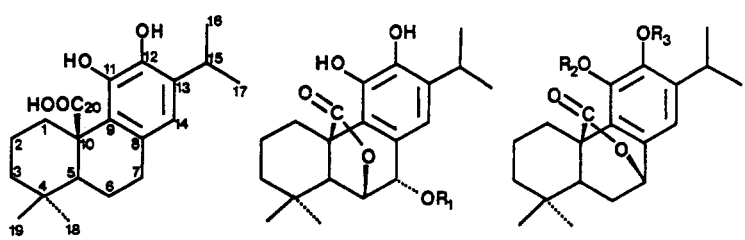
RESULTS AND DISCUSSION

Compound **1** was identified as carnosolic acid ($C_{20}H_{28}O_4$, Mr= 332.1992, calcd 332.1988) by comparison of its spectral data with published values (13). This is the first report of direct isolation of carnosolic acid from plant material. Compounds **2**, **3**, **4**, **5**, and **6** were identified as rosmanol, 7-*O*-methylrosmanol, 7-*O*-ethylrosmanol, carnosol, and 11,12-*O,O*-dimethylcarnosol, respectively. Their structures were confirmed on the basis of physical and spectroscopic data previously reported (13,22,23).

In the first set of experiments, the compounds were tested against HIV-1 protease activity. The results are shown in Table 1. Interestingly, the carnosolic acid showed the strongest inhibitory activity (IC_{90}=0.08 µg/ml).

Additionally, the carnosolic acid was assayed versus the HIV-1 virus replication. The IC_{90} value (0.32 µg/ml) reveals that the carnosolic acid displayed a potent antiviral effect.

In order to determine the cytotoxicity of carnosolic acid, the influence of compound **1** on cell growth was tested. The antiviral activity of carnosolic acid against HIV-1 virus is not significant, since we found the cytotoxic TC_{90} is 0.36



1

2 R_1=H
3 R_1=Me
4 R_1=CH_2CH_3

5 R_2=R_3=H
6 R_2=R_3=Me

TABLE 1. Inhibition of HIV-1 Protease by Tested Compounds.

Compound	IC_{90} (μg/ml)
Carnosolic acid [**1**]	0.08
Rosmanol [**2**]	0.600
7-*O*-Methylrosmanol [**3**]	1.5
7-*O*-Ethylrosmanol [**4**]	1.7
Carnosol [**5**]	>10
11,12-*O,O*-Dimethylcarnosol [**6**]	>10

μg/ml (on H9 lymphocytes in culture). However, the strong inhibition of the viral protease suggests carnosolic acid derivatives may be found which are selectively antiviral.

HIV-1 protease belongs to the group of aspartic proteases; therefore the inhibitory effect of all six substances against cellular aspartic proteases cathepsin D and pepsin was assayed. At the concentration range up to 10 μg/ml, the tested compounds did not inhibit proteolytic activity of cathepsin D nor pepsin.

Comparison of the structure-activity relationship between carnosolic acid and its naturally occurring derivatives carnosol and rosmanol reveals that the planar aromatic ring with two ortho-phenolic groups, which are important for some biological activities of these compounds, are probably not involved directly in inhibition of HIV-1 protease. Moreover, besides the free carboxylic group the most important difference between compounds **1**, **2**, and **5** is the benzylic CH_2 group at position 7 in the structure of carnosolic acid [**1**], which would be expected to be very reactive and readily oxidizable. The very reactive intermediate formed in the first step of oxidation of carnosolic acid might react with a nucleophilic center of HIV protease. The cytotoxicity of carnosolic acid, which is very close to its effective antiviral dose, supports such a proposition. Additionally, HIV-1 activity assay and cytotoxicity studies were performed at nearly neutral pH (7.2), where carnosolic acid can be readily oxidized. On the other hand, other tests were measured at pH 3.5 and 1.8, respectively, where carnosolic acid is relatively stable. In the case of carnosolic acid derivatives, the stable intramolecular lactone ring is formed as a result of spontaneous oxidation of the benzylic methylene group of carnosolic acid in polar solvents (compounds **2**, **3**, **4** at position 6 and compound **5** at position 7) (13). The greater antiviral activity of γ-lactones (compounds **2**, **3**, and **4**) compared to carnosol [**5**] can be explained by higher reactivity of a substituted benzylic group at position 7, which may undergo further oxidation or substitution. However, the exact mechanism of the interaction still remains to be elucidated.

The present study could be a starting point to design a less cytotoxic agent which might have application in the chemotherapy of AIDS.

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LITERATURE CITED

1. J. Kay and B.M. Dunn, *Biochim. Biophys. Acta,* **1048**, 1 (1990).
2. R.C. Gallo and L. Montagnier, *Sci. Am.,* **259**, 25 (1988).
3. L.H. Pearl and W.R. Taylor, *Nature,* **329**, 351 (1987).
4. M.A. Navia, P.M.D. Fitzgerald, B.M. McKeever, C.T. Leu, J.C. Heimbach, W.K. Herber, I.S. Sigal, P.L. Darke, and J.P. Springer, *Nature,* **337**, 615 (1989).
5. S. Seelmeier, H. Schmidt, V. Turk, and K. von der Helm, *Proc. Natl. Acad. Sci. USA,* **85**, 6612 (1988).
6. N.E. Kohl, E.A. Emini, W.A. Schleif, L.J. Davis, J.C. Heimbach, R.A.F. Dixon, E.M. Scolnick, and I.S. Sigal, *Proc. Natl Acad. Sci. USA,* **85**, 4686 (1988).
7. H.G. Krausslich, S. Oroszlan, and E. Wimmer, Eds., "Viral Proteinases as Targets for Chemotherapy," Cold Spring Harbour Laboratory Press, New York, 1989.
8. P.M.D. Fitzgerald and J.P. Springer, *Annu. Rev. Biophys. Biophys. Chem.,* **20**, 299 (1991).
9. A.D. Richards, R. Roberts, B.M. Dunn, M.C. Graves, and J. Kay, *FEBS Lett.,* **247**, 113 (1989).
10. E. Sarubbi, M.L. Nolli, F. Andronico, S.

Stella, G. Saddler, E. Selva, A. Siccardi, and M. Denaro, *FEBS Lett.*, **279**, 265 (1991).
11. R.I. Brinkworth, M.J. Stoermer, and D.P. Fairlie, *Biochem. Biophys. Res. Commun.*, **188**, 631 (1992).
12. G. Buchbauer, H. Spreitzer, and G. Kiener, *Pharm. Unserer Zeit*, **19**, 24 (1990).
13. E. Wenkert, A. Fuchs, and J.D. McChesney, *J. Org. Chem.*, **30**, 2931 (1965).
14. B. Korant and C. Rizzo, *Biol. Chem. Hoppe-Seyler*, **373**, 517 (1992).
15. Y. Cheng, M. McGowan, C. Kettner, J. Schloss, S. Erickson-Viitanen, and F. Yin, *Gene*, **87**, 243 (1990).
16. S. Erickson-Viitanen, J. Manfredi, P. Viitanen, D. Tribe, R. Trich, C. Hutchinson, D. Loeb, and R. Swanstrom, *AIDS Res. Hum. Retroviruses*, **5**, 577 (1989).
17. R. Smith and V. Turk, *Eur. J. Biochem.*, **48**, 245 (1974).
18. S. Harada, Y. Koyawagi, and N. Yamamato, *Science*, **229**, 563 (1985).
19. H. Nakashima, R. Pauwels, M. Baba, D. Schols, J. Desmyter, and E. DeClerq, *J. Virol. Methods*, **26**, 918 (1989).
20. D. Scudiero, R. Shoemaker, K. Paull, A. Monks, S. Tierney, T. Nofziger, M. Gurrens, D. Seniff, and M. Boyd, *Cancer Res.*, **48**, 4827 (1988).
21. M.L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).
22. R. Inatani, N. Nakatani, H. Fuwa, and H. Seto, *Agric. Biol. Chem.*, **46**, 1661 (1982).
23. N. Nakatani and R. Inatani, *Agric. Biol. Chem.*, **48**, 2081 (1984).

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