L-5-Hydroxytryptophan: Antioxidant and Anti-Apoptotic Principle of the Intertidal Sponge *Hymeniacidon heliophila*

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The intertidal sponge *Hymeniacidon heliophila* which survives under intense sunlight contains the antioxidant amino acid L-5-hydroxytryptophan (L-5-HTP) as major constituent. The content of L-5-HTP was determined as (0.45 ± 0.23)% of the dry weight by quantitative NMR-spectroscopical analysis with an internal standard. Other known antioxidants such as flavonoids, carotenoids or tocopherol derivatives were absent. Both the oxidation potential and the concentration of L-5-HTP in *H. heliophila* correspond to the values observed for flavonoids being major antioxidants in plants. It was shown that L-5-HTP suppresses UV-induced apoptosis in human monocytes at the same concentrations as it occurs in the sponge tissue.

Key words: Antioxidant, Apoptosis, Marine sponge

Introduction

Oxidative stress is caused by the reaction of chemically aggressive oxygen species with biomolecules and contributes to the development of aging, atherosclerosis, cancer, or Alzheimer's disease (Kannan and Jain, 2000). In plants, phenolic compounds and carotinoids constitute the major tissue-protecting antioxidants (Jovanovic *et al.*, 1994). Much less is known on antioxidants from animals (Potterat, 1997). Sessile marine animals from tropical and subtropical regions appear to be especially interesting, because they live directly below the water surface heavily exposed to intense sun light (Dunlap *et al.*, 1999). An example is the "sun-loving" sponge *Hymeniacidon heliophila* occurring in the intertidal regions along the coastline of North Carolina (USA). We became curious about the existence of a chemical reason for the prevention of oxidative damage of the sponge. Furthermore, it was an intriguing question whether a constituent of the extract of *H. heliophila* could be of use for the protection of human cells, *e. g.*, against UV-induced apoptosis. Biological function gains importance in natural products research.

Experimental

General

Column chromatography was carried out on Sephadex LH-20 (Pharmacia). Thin-layer chromatography (TLC) was performed RP-18 silica gel (F_{254} Merck). The EI mass spectra were recorded on a JEOL JMS-700 mass spectrometer. The optical rotation was recorded using a Perkin-Elmer 241 A polarimeter with a 10 cm cell. Human blood buffys were provided by the blood bank of the University of Heidelberg from healthy volunteers. RPMI-1640 medium with indicator was obtained from BRL Life Technologies, Eggenstein, and supplemented with L-glutamine (2 mM), penicillin ($50\ U \cdot l^{-1}$), streptomycin ($50\ \mu g \cdot l^{-1}$), and fetal calf serum (10%, inactivated at 56 °C, 30 min), followed by sterile filtration (0.22 μm). The medium was stored at 4 °C and used within 7 days. Fetal calf serum (FCS) was purchased from Roche Diagnostics, Mannheim. Lymphocyte separation medium was purchased from PAA, Linz, Austria.

Collection

The specimens of the sponge *Hymeniacidon heliophila* were collected from light and dark areas of the intertidal zone at the coast of Wilmington

(North Carolina, USA, 34 N 77 W, July 1999) and lyophilized immediately.

Identification of L-*5-hydroxytryptophan* (**1**)

A sample of the freeze-dried sponge *Hymeniacidon heliophila* (231 g) was extracted with MeOH/CH_2Cl_2 (1:1, 1500 ml, 3 times). After concentration the crude extract (34.5 g) was partitioned between isooctane and MeOH. The MeOH phase was washed with isooctane (3 times) and concentrated. The residue was partitioned between *n*-BuOH and water. The *n*-BuOH phase (4.7 g) was washed with water and a portion (2.6 g) was fractioned by gel chromatography (Sephadex LH-20, MeOH column length 1 m, column diameter 6 cm). A pure sample of L-5-HTP (**1**, 62 mg) was collected; TLC: R_f = 0.76 (RP-18, MeOH/H_2O (1:1)); $[\alpha]_D^{20}$ = – 27.4, (c = 5.63 mM in H_2O); ^{1}H NMR (400 MHz, chloroform-d_1/methanol-d_4 (4:6 v/v)): δ = 7.22 (d, J = 8.8 Hz, 1H, 7-H), 7.13 (s, 1H, 2-H), 7.06 (d, J = 2.4 Hz, 4-H), 6.73 (dd, J = 2.4, 8.8 Hz, 1H, 6-H), 3.83 (dd, J = 9.6, 4.0 Hz, 2′-H), 3.46 (dd, J = 15.2, 3.8 Hz, 1H, 3′-H), 3.09 (dd, J = 15.2, 9.6 Hz, 3′-H); ^{13}C NMR (125 MHz, methanol-d_4): δ = 174.7 (C-1′), 151.5 (C-5), 133.2 (C-7a), 129.1 (C-3a), 125.9 (C-2), 112.9 (C-6), 112.8 (C-7), 108.7 (C-3), 103.5 (C-4), 56.5 (C-2′), 28.5 (C-3′); MS (EI, 70 eV): m/z (%) = 220 $[M]^+$; HREIMS: $C_{11}H_{12}N_2O_3$ $[M]^+$: calcd. 220.0845: found 220.0863.

Quantification of L-*5-hydroxytryptophan* (**1**)

Quantitative extractions of freeze-dried specimens of *Hymeniacidon heliophila* (2.53 to 5.92 g, see Table I) were performed using water/EtOH (1:1) and monitored by TLC. An aqueous solution of maleic acid (10 ml, 1 mg·ml^{-1}) was added to each of the extracts (ranging from 0.75 to 1.56 g). After concentration to dryness, the mixtures were completely dissolved in D_2O/CD_3OD (1:1 v/v). ^{1}H NMR spectra of 1-ml portions were taken and analyzed by integration.

Isolation and culture of human monocytes

Human blood buffy (30 ml) was overlayered with lymphocyte separation medium (20 ml, 1.078 g·ml^{-1}). After centrifugation (1000 g, 30 min), the monocytes were collected from the banded plasma-LSM interphase and washed/centrifuged three times with isotonic phosphate buffer (pH 7.4). The cells were then suspended in RPMI 1640 medium, counted (Neubauer chamber, trypan blue dye exclusion) and diluted to a density of $7.5 \cdot 10^5$ cells per ml RPMI 1640 medium. The monocytes were then seeded in 24 well tissue plates (1 ml per well, 37 °C, 2 d, 5% CO_2, steam atmosphere). After 2 days the medium was removed and the adhering monocytes were washed with phosphate buffer (pH 7.4).

Table I. Contents of L-5-HTP in five specimens of *Hymeniacidon heliophila* as determined by integration of ^{1}H NMR signals using maleic acid as internal standard. In addition, the average over all samples is given.

Specimen	Content of 5-HTP [%][a]	Conc. [mM][b]	Light exposure
1	0.49 ± 0.06	2.2 ± 0.3	sun
2	0.36 ± 0.01	1.6 ± 0.1	sun
3	0.57 ± 0.06	2.6 ± 0.3	shadow
4	0.48 ± 0.03	2.2 ± 0.2	sun
5	0.32 ± 0.01	1.5 ± 0.1	shadow
Average	0.45 ± 0.23	2.0 ± 1.0	–

[a] Referred to the dry weight; calculated from the ratio of the average integration of the ^{1}H NMR signals of the aromatic protons of L-5-HTP and the internal standard maleic acid (concentration 1 mg·ml^{-1}); [b] based on a density of 0.1 mg·ml^{-1} of the dry sponge.

Determination of the inhibition of UV-induced apoptosis by L-*5-HTP*

Appropriate solutions of the test compounds (L-5-HTP, acetylcysteine) in phenol red-free RPMI 1640 medium (0.5 ml) were added to the pellet and incubated at 37 °C for 30 min. The wells were irradiated with UV light (λ = 254 nm, 1 mW·cm^{-2}, distance of the lamp 10 cm) at 37 °C for 2 h. Rates of apoptotic cells were determined as described (Deigner *et al.*, 2001): YOPRO-1 iodide (14 μl, 0.1 mM in DMSO/phosphate buffer) and Hoechst 33342 (10 μl, 0.1 mM in DMSO/phosphate buffer) were then added. After 20 min, the fluorescent nuclei were counted employing the corresponding filters (340–380 nm *resp.* 450–490 nm).

Results

To investigate the presence of other major antioxidants in the sponge, an exhaustive extraction of the freeze-dried organism was performed with the solvent mixture dichloromethane/methanol (1:1). Fig. 1 gives the ^{1}H NMR spectra of the MeOH/CH_2Cl_2 (1:1) extract of the lyophilized sponge *Hymeniacidon heliophila* and of the purified compound. L-5-HTP (**1**) is the single major constituent showing signals in the olefinic/aromatic region. Differences in the ^{1}H NMR chemical shifts of the L-5-HTP (**1**) signals arise from slightly different ra-

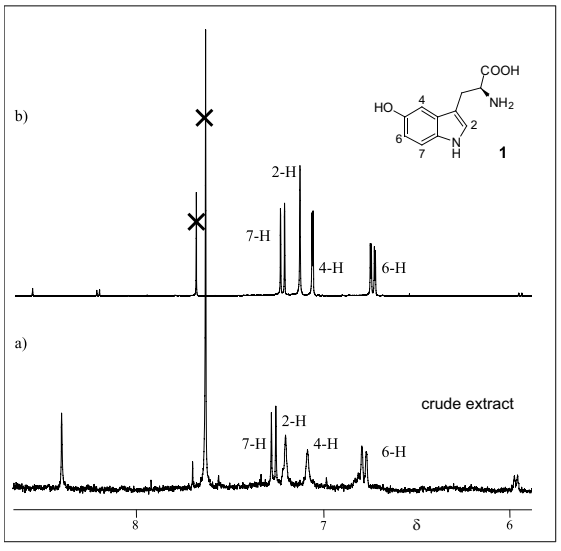

Fig. 1. ^{1}H NMR spectrum of a) the crude, exhaustive extract (dichloromethane/methanol (1:1 v/v)) of *Hymeniacidon heliophila* in chloroform-d_1/methanol-d_4 (50:50 v/v). The aromatic region of the ^{1}H NMR spectrum of b) the isolated L-5-hydroxytryptophan (**1**) is given for comparison (chloroform-d_1/methanol-d_4 (40:60 v/v)). Solvent signals ($CHCl_3$) show different chemical shifts, because different mixtures with methanol-d_4 have been used.

tios of the deuterated solvents chloroform-d_1 and methanol-d_4. The ^{1}H NMR signals were unambigously assigned on the basis of COSY, HSQC, and HMBC spectra. The isomer 6-hydroxytryptophan could be excluded by ^{13}C NMR chemical shift and HMBC analysis. Comparison of the specific optical rotations of the isolated (– 27.4) and of L-5-HTP (– 29.8) assigns the absolute configuration 2′*S* to **1**. Although L-5-HTP is the biosynthetic precursor of serotonin and melatonin, used as their prodrug in anti-depressive therapy, the latter metabolites were not detected as constituents of the extract of *H. heliophila*.

For quantitative analysis, lyophilized samples of *H. heliophila* were extracted exhaustively (controled by thin layer chromatography) with ethanol/water (1:1 v/v). For each of the five specimens, two extracts were prepared and subsequently analyzed by ^{1}H NMR integration. Table I gives the contents of L-5-HTP (**1**) in five different specimens of *H. heliophila* as determined by comparative integration of the least intense indole ^{1}H NMR signal (δ 6.79, δ 7.05, δ 7.27, or δ 7.33) with the signal at δ 6.35 of the two double bond protons of the internal standard maleic acid in CD_3OD/D_2O (1:1). The average L-5-HTP content of *H. heliophila* was determined as (0.45 ± 0.23) % of the dry weight. The quantification by NMR had to take into account the large quantities of sodium chloride as it is typical for marine organisms. Therefore, the NMR solvent mixture methanol-d_4/water-d_2 was used which completely dissolved the polar extract. It was prevented that any L-5-HTP would remain attached to sodium chloride insoluble in other NMR solvents.

Table II and Fig. 2 give the results of comparative bioassays for inhibition of apoptosis in human monocytes. The monocytes were prepared from human blood by removal of the plasma, separation of the lymphocytes, washing and resuspension steps, and dissemination in 24-well plates (RPMI 1640 medium).

Table II. Inhibition of apoptosis in human monocytes by different concentrations of L-5-HTP (**1**). For comparison, *N*-acetylcysteine (ACC) was tested.

Conditions	Degree of apoptosis [%][a]
no UV, 0.0 mM 5-HTP	21 ± 10 (12)
no UV, 10 mM 5-HTP	17 ± 8 (11)
UV, 10 mM 5-HTP	18 ± 8 (10)
UV, 5.0 mM 5-HTP	19 ± 10 (12)
UV, 1.0 mM 5-HTP	50 ± 21 (12)
UV, 0.5 mM 5-HTP	60 ± 22 (12)
UV, 0.0 mM 5-HTP	62 ± 17 (11)
UV, 5.0 mM ACC	48 ± 20 (9)

[a] Numbers of experiments are given in brackets.

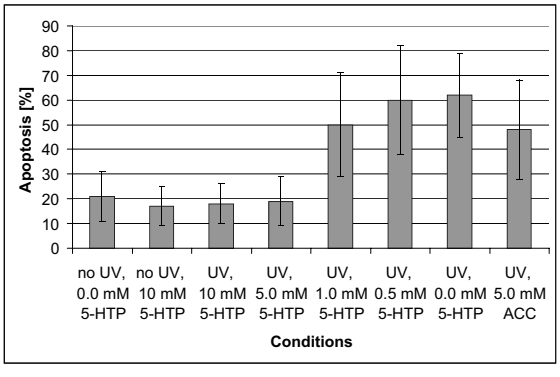

Fig. 2. Apoptosis [%] of UV-exposed (λ = 254 nm, 1 mW·cm^{-2}, 2 h) human monocytes in the presence of different concentrations of L-5-HTP (**1**). ACC: *N*-acetylcysteine. Standard deviations are given, according to Table II.

The assays were performed after 2 days of equilibration. For induction of oxidative stress, cells were exposed to UV light (λ = 254 nm, 1 mW·cm^{-2}) for 2 h. For analysis, the monocytes were treated with the dye Hoechst 33342 and incubated with YO-PRO 1 which exclusively stains apoptotic cells (Idziorek *et al.*, 1995). Cells were counted under a light microscope using appropriate fluorescence filters. In a series of three independant experiments, four concentrations of 5-HTP (**1**, 10 mM, 5 mM, 1 mM, 0.5 mM) and, for comparison, the known anti-apoptotic agent *N*-acetylcysteine (ACC, 5 mM; Slyshenkov *et al.*, 2001) were tested (the compounds were dissolved in RPMI 1640 medium without phenol red). The other columns (Fig. 2) refer to control experiments.

At 10 mM and 5 mM concentrations of L-5-HTP the protection of the human monocytes against UV-induced apoptosis is pronounced. At 1 mM concentrations of L-5-HTP, UV-induced apoptosis is still somewhat inhibited ((50 ± 21)% apoptosis) in comparison to the control ACC (5 mM, (62 ± 17)%). At 0.5 mM, no inhibition can be observed.

Discussion

L-5-Hydroxytryptophan is an antioxidant. Following the discovery of hydroxyindoles as a new class of potential endogenous antioxidants, Jovanovic *et al.* (1990) determined that the one-electron redox potential of 5-HTP ranges from E = 0.29 V at pH 13.5 to E = 0.86 V at pH 3. At pH 7, 5-HTP is oxidized (E = 0.64 V), *e.g.*, by the radicals of tyrosine or guanine and, thereby, is able to prevent damage of biochemically important molecules at physiological pH. 5-HTP and the flavonoids quercetin and kaempferol (E = 0.60 V, 0.95 V; Jovanovic *et al.*, 1994) show comparable one-electron redox potentials and, therefore, may serve similar antioxidant functions (for a review on radical functions *in vivo*, see Saran *et al.* (1998). The mechanism of the reaction of the 5-hydroxyindole serotonin with hydroxyl radicals has been studied (Hela *et al.*, 1999).

Recent studies have shown that oxidative stress predominantely causes apoptosis (Hampton and Orrenius, 1997) which can be diminished by antioxidants such as *N*-acetylcysteine (Buttke and Sandstrom, 1994). The content of L-5-HTP in the tissue of the freeze-dried sponge *Hymeniacidon heliophila* ((0.45 ± 0.23)% weight) corresponds to an estimated concentration between 1 mM and 2 mM. Therefore, concentrations ranging from 0.5 to 10 mM, comparable to the situation in the sponge tissue, were chosen in our experiments on the inhibition of apoptosis in human monocytes.

Indeed, the investigated monocytes showed a high degree of apoptosis (62%) when irradiated with short-wave UV light for 2 h in the control experiment. The standard deviations are within the expected range, if it is taken into account that the monocytes are from different persons and that rates of apoptosis are genetically regulated. As soon as UV light was excluded, cells showed comparably low rates of apoptosis under the test conditions both in the presence ((17 ± 8)%, 10 mM) and the absence ((21 ± 10)%) of L-5-HTP (**1**). At 5-HTP concentrations of 10 mM (18 ± 8)% apoptosis) and 5 mM ((19 ± 10)%), the rate of UV-induced apoptosis was much lower than when *N*-acetylcysteine was used at 5 mM ((48 ± 20)% apoptosis). In fact, the inhibition at 10 mM and 5 mM brought apoptosis to the level of the control which was not irradiated. It can be concluded that 5-HTP is more active than the standard ACC with regard to the inhibition of apoptosis in human monocytes. At 10 mM, L-5-HTP is not toxic to the investigated human monocytes and prevents apoptosis. The low molar absorption coefficient of 5-HTP at λ = 254 nm (ε = 1852 mol^{-1}·l·cm^{-1}) excludes the possibility that absorption rather than antioxidant properties are reponsible for the cell protection.

Requirements for L-5-HTP being the antioxidant principle of *H. heliophila* include that (a) the compound is present in the concentration range of other well-known antioxidants like flavonoids in plants and (b) other compounds possibly serving that function are absent or very minor. The average concentration of antioxidant flavonoids in plants (0.5–1.5%) is within the same range as the average content of 0.45% of L-5-HTP found in *H. heliophila*, sampled over specimens collected at different locations. Based on the absence of other antioxidants, it is concluded that L-5-HTP is the antioxidant principle of *H. heliophila*.

Our study for the first time gives an animal example supporting the hypothesis by Jovanovic *et al.* (1990) proposing the function of 5-HTP as a naturally occurring, endogenous antioxidant. Few

small-molecule antioxidants have been identified from marine organisms so far (Sakata *et al.*, 1994). Earlier, 5-HTP had been found only once in high concentration as an insect repellent in the seeds of the medicinal plant *Griffonia simplicifolia* (Bell and Fellows, 1966). Oxidized tryptophan metabolites have been proposed as agents against inflammatory diseases (Christen *et al.*, 1990).

A recent publication (Betten *et al.*, 2001) reported a protective and scavening effect of serotonin as well as of 5-HTP in reactive oxygen species dependent apoptogenesis in natural killer cells (NK-cells), a subpopulation of human mononuclear cells, by a yet not well characterized mechanism. The contribution of the scavenging activity of these compounds on the immunoregulatory network remains still unclear. Culturing sponge cells has not yet become generally possible, although initial success has been reported (Custodio *et al.*, 1998, De Rosa *et al.*, 2001). It would be the next step to study the cells of *H. heliophila.*

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