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A Novel Type of Nonsteroidal Estrone Sulfatase Inhibitors

Peter Jütten,* Winfried Schumann, Albert Härtl, Lothar Heinisch, Udo Gräfe,* Walter Werner and Hermann Ulbricht

Hans-Knöll Institute of Natural Products Research, Beutenbergstrasse 1, 07745 Jena, Germany

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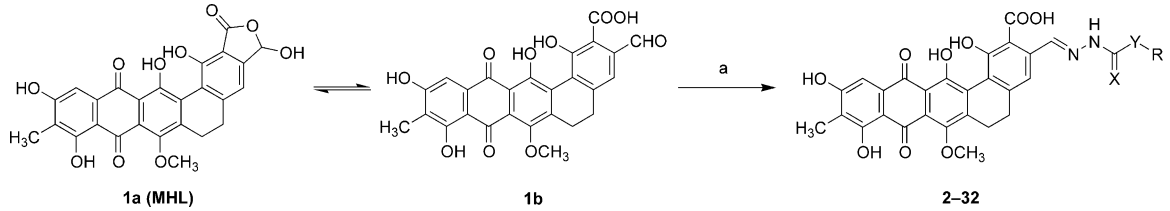
Abstract—Madurahydroxylactone (MHL) is a secondary metabolite produced by the soil bacterium *Nonomuria rubra* and belongs to the family of benzo[*a*]naphthacenequinones. We report the initial results and structure–activity relationships of our study into a series of thiosemicarbazone derivatives of madurahydroxylactone as potential nonsteroidal inhibitors of the enzyme estrone sulfatase. The most active compound, the cyclohexylthiosemicarbazone, was shown to be a non-competitive inhibitor with a K_i of 0.35 μM. This compound is devoid of estrogenic properties and showed low acute toxicity in the hen's fertile egg screening test.

About one-third of breast cancers are classified as estrogen-dependent. Numerous reports have suggested the importance of estrone sulfate and estrone sulfatase in regulating the supply of estrogens to these cancers. Therefore, inhibition of estrone sulfatase is an important target for the development of new drugs for the treatment of these diseases.

The very first inhibitors of estrone sulfatase were steroidal compounds, the most potent of which was estrone-3-*O*-sulfamate (EMATE).[1] The steroid nucleus, however, presented complications, in that EMATE was found to be an irreversible inhibitor which releases estrone during enzyme inactivation[2] and possesses strong estrogenic properties.[3] A number of strategies have therefore been adopted to design and synthesize inhibitors based on a nonsteroidal structure[4–9] because such compounds are less likely to be converted to metabolites with hormonal activity. In general, these inhibitors are phenol sulfamate esters, for example, 4-methylcoumarin-7-*O*-sulfamate (COUMATE), that mimic the A-ring of estrone whereas the side chain(s) provide(s) a lipophilic bulk mimicking the B-C-D skeleton of steroid substrates.[4]

We have developed and synthesized a series of novel nonsteroidal compounds derived from the natural product madurahydroxylactone (**1a**, Scheme 1) that are potent estrone sulfatase inhibitors. Madurahydroxylactone (MHL) is a secondary metabolite produced by the soil bacterium *Nonomuria rubra*. The red coloured compound was already isolated in the seventies,[10] however, its complex structure has only recently been established to be a highly substituted benzo[*a*]naphthacenequinone by X-ray single crystal structure determination: 3,9,11,14,15-pentahydroxy-7-methoxy-10-methyl-1,8,13-trioxo-1,3,5,6,8,13-hexahydronaphthaceno[1,2-*f*]-isobenzofuran.[11]



Scheme 1. Synthesis of madurahydroxylactone thiosemicarbazones and other *N*-acyl-hydrazones [a = R-Y-C(X)-NHNH$_2$, X = O, S; THF, rt or Δ].

*Corresponding author. Fax: +49-3641-65-6705; e-mail: pjuetten@t-online.de; ugraefe@pmail.hki-jena.de

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Although only a small group of compounds, benzo[*a*]-naphthacenequinones have a wide spectrum of biological activities: the benanomicins[12] and pradimicins[13,14] are prominent antifungal and antiviral agents; other compounds show antibacterial activity or are inhibitors of enzymes like α-glucosidase, endothelin converting enzyme, glutathione S-transferase, or calmodulin-dependent cyclic nucleotide phosphodiesterase.[15]

As madurahydroxylactone exhibits an interesting but insufficient activity against Gram-positive bacteria, a derivatization programme was started providing compounds with promising antibacterial activities.[15,16] With the broad biological activity profile of the benzo[*a*]-naphthacenequinones in mind we were not surprised to obtain MHL derivatives with antimycobacterial activity, cytotoxic compounds, as well as inhibitors of several enzymes, for example, gyrase, topoisomerase I/II, glutathione S-transferase, endothelin converting enzyme, phosphotyrosinyl phosphatase, PI3 kinase, as well as inhibitors of cytokine release, and estrone sulfatase. Herein, we report the initial results of our study where we have undertaken the synthesis of thiosemicarbazone and other acylhydrazone derivatives of madurahydroxylactone, the in vitro biochemical evaluation of the synthesized compounds, and further tests for estrogenicity and acute toxicity of the most potent inhibitor.

Chemistry

Madurahydroxylactone (**1a**, Scheme 1) bears a phthalide part. In acidic and neutral medium the compound typically exists as this intramolecular hemiacetal that is insoluble in water. MHL readily dissolves, however, in alkaline medium forming salts of an *ortho*-formylcarboxylic acid (**1b**). When MHL is treated with hydrazine, alkyl- or arylhydrazines, naphthaceno[1,2-*g*]phthalazines are formed.[16] Hydrazides differ in their reaction from that of the hydrazines. Thiosemicarbazides, semicarbazides, and other hydrazides of carbonic acid derivatives readily reacted with madurahydroxylactone to yield the corresponding hydrazones of the aldehyde in boiling tetrahydrofuran solvent or at room temperature (Scheme 1, Table 1). Thiosemicarbazones and related derivatives of MHL were easily obtained in good yields. The appropriate reagents are commercially available or were prepared by literature procedures without any major problems.[17] The synthesis of madurahydroxylactone cyclohexylthiosemicarbazone (**18**, 3-(4-cyclohexylthiosemicarbazono)methyl-1,9,11,14-tetrahydroxy-7-methoxy-10-methyl-8,13-dioxo-5,6,8,13-tetrahydrobenzo[*a*]naphthacene-2-carboxylic acid) is given as an example.

Madurahydroxylactone Cyclohexylthiosemicarbazone (18)

4-Cyclohexylthiosemicarbazide (4.77 g, 27.5 mmol) in one portion was added to a boiling solution of **1** (15.00 g, purity 90%, 27.5 mmol) in THF (700 mL). The reaction mixture was cooled to room temperature, diluted with THF (700 mL) and filtered. The filtrate was concentrated to one-third of the original volume. Hexane (1000 mL) was added dropwise to the well-stirred concentrate to give **18** as a red crystalline compound which was filtered off and dried in vacuo (17.61 g, 99%). ^{1}H NMR (d_6-DMSO) δ 1.11–1.98 (m, 10H), 2.06 (s, 3H), 2.69–2.91 (m, 4H), 3.79 (s, 3H), 4.07–4.25 (m, 1H), 7.26, 7.44 (2 s, 2H), 7.85 (d, $J = 8.5$ Hz, 1H), 8.39 (s, 1H), 11.50 (s, 1H), 13.56 (s, 1H). ^{13}C NMR (d_6-DMSO) δ 8.15, 22.42, 25.08, 28.74, 24.76, 31.80, 52.53, 61.05, 106.36, 117.23, 109.69, 114.15, 118.41, 119.30, 120.18, 122.20, 129.68, 130.71, 133.32, 143.12, 146.52, 150.75, 154.63, 156.11, 162.01, 162.42, 169.95, 175.85, 140.60, 185.52, 187.58. FABMS *m*/*z* 646 $(C_{33}H_{31}N_3O_9S)H^+$.

Estrone Sulfatase Assay

^{3}H-Estrone sulfate (500,000 dpm/tube) adjusted to 20 μM with unlabeled estrone sulfate E_1S in Tris–HCl buffer (0.2 M, pH 8.0, 0.1 mL) was added to a test tube. An inhibitor at various concentrations in Tris–HCl buffer (0.1 mL) was then added to each tube. The assay began by the addition of placental microsomes diluted with Tris–HCl buffer containing 2.5 mM dithiothreitol (0.3 mL). The protein concentration was measured using the Bradford method. The final volume of the assay was 0.5 mL. After 20 min of incubation at 37 °C, 1.5 mL of toluene was added to quench the assay. Control samples

Table 1. Inhibition data for madurahydroxylactone **1** and synthesized compounds **2**–**32**, as well as EMATE and COUMATE

Compd	X	Y	R	IC_{50} (μM)
1	—	—	—	41
2	S	NH	H	> 100
3	S	NH	CH_3	29
4	S	NH	C_2H_5	16
5	S	NH	*n*-C_3H_7	20
6	S	NH	*n*-C_4H_9	6
7	S	NH	*n*-C_5H_{11}	13
8	S	NH	*n*-C_6H_{13}	8
9	S	NH	*n*-C_7H_{15}	9
10	S	NH	*n*-C_9H_{19}	19
11	S	NH	*n*-$C_{12}H_{25}$	50
12	S	NH	*i*-C_3H_7	24
13	S	NH	*i*-C_4H_9	11
14	S	NH	*t*-C_4H_9	25
15	S	NH	$CHEt_2$	18
16	S	NH	*c*-C_3H_5	22
17	S	NH	*c*-C_5H_9	3.2
18	S	NH	*c*-C_6H_{11}	0.46
19	S	NH	*c*-C_7H_{13}	1.8
20	S	NH	*c*-C_8H_{17}	15
21	S	NH	Ph	4.8
22	S	NH	4-Cl-Ph	> 100
23	S	NH	4-Me-Ph	12
24	S	NH	4-MeO-Ph	23
25	S	NH	4-Me-*c*-C_6H_{10}	4.0
26	S	NCH_3	*c*-C_6H_{11}	1.4
27	S	NH	Piperidino	6
28	S		Piperidino	8
29	O	NH	*c*-C_6H_{11}	7
30	O	NH	Ph	> 100
31	S	O	Ph	> 100
32	O	O	Ph	> 100
EMATE	—	—	—	0.08
COUMATE	—	—	—	2.6

without inhibitor were incubated simultaneously. The quenched samples were vortexed for 1 min. Fifty microlitres of the toluene phase was removed and diluted with 3 mL of scintillation cocktail. The aliquots were counted for 3 min to determine the amount of product formation. All samples were run twice in duplicate. Product formation for samples containing an inhibitor was compared to that of the control sample.

Kinetic Analysis

Estrone sulfatase assays were carried out as described above using various concentrations of the substrate (2–20 μM). The K_m, K_i, and type of inhibition for **18** at pH 8.0 were determined by Lineweaver–Burk plot analysis (Fig. 1A) and secondary replots of the slopes of Lineweaver–Burk plot versus the corresponding inhibitor concentration (0, 0.5, 1, 2.5, 5 μM; Fig. 1B).

We started a limited exploration of the structure–activity relationships of MHL thiosemicarbazones. The structures of the new synthesized compounds and the results of the estrone sulfatase assay of MHL (**1**) and its derivatives (**2**–**32**) are shown in Scheme 1 and Table 1. The sulfamates EMATE and COUMATE were tested as standard compounds in our assay for comparison with literature data. We found that all *n*-alkylthiosemicarbazones (**3**–**11**) exhibited increased estrone sulfatase inhibition compared to the parent compound (**2**) and madurahydroxylactone (**1**). The IC_{50}s of the most potent member, the butyl isomer **6**, was 6 μM. In every instance the *n*-alkyl members were slightly more potent than the isoalkyl isosters (**12**–**15**). Encouraged by these results, we investigated cyclic substituents. The cycloalkylthiosemicarbazones are potent inhibitors of estrone sulfatase. One of them, namely, the cyclohexylthiosemicarbazone **18** has the highest activity and a IC_{50} value of 460 nM, being only 5.8 times weaker than EMATE. In comparison to COUMATE, we observe that the cyclopentyl (**17**), cycloheptyl (**19**), and cyclohexyl compounds (**18**) are equipotent or much stronger inhibitors than COUMATE. The phenylthiosemicarbazone (**21**) is about 10-fold less potent than **18**. Introduction of *para*-substituents in the phenyl moiety (**22**–**25**) proved to be unfavorable. We focused our further studies on the lead compound **18**, designing the following structural modifications: addition of methyl to the cyclohexyl group (**25**) or the vicinal nitrogen (**26**), replacement of the cyclohexyl *ipso* carbon with a nitrogen (**27**), and incorporation of the thiocarbamoyl nitrogen in the ring (**28**). All these analogues are 3–17 times less in affinity to the sulfatase than inhibitor **18**. Analogues of **18** and **21** were synthesized in which the hetero atoms in the thiocarbaminic acid part are replaced by oxygen or sulfur (**29**–**32**). All replacements dramatically reduced the activity. The results confirm that the thiosemicarbazono functionality is favorable for sulfatase inhibitory activity.

On this basis, further studies were undertaken to investigate the pharmacological characeristics of **18** as a new prototypic estrone sulfatase inhibitor. It was to be expected that the compound is not an active-site directed inhibitor. Indeed, in vitro kinetic studies revealed **18** to be a non-competitive inhibitor of estrone sulfatase (Fig. 1). For clinical utility, estrone sulfatase inhibitors must not be estrogen agonists, therefore these compounds were also assessed for estrogenic properties in vivo. Compound **18** was administered to ovariectomized adult female Wistar rats (single dose of 50 mg/kg po and sc), two weeks after ovariectomy had been performed. Administration did not result in any increase in the uterine weight nor did it cause any cornification of the vagina, showing that thiosemicarbazone **18** is devoid of any estrogenic effect. The acute toxicity of **18** was studied in the hen's fertile egg screening test (HEST). Fifteen-day-old chick embryos receive the test compound through the air cell and deaths were measured at 72–96 h after the treatment. The LD_{50} of HEST is strongly positively correlated to iv LD_{50} obtained in mice and rodents.[18] The acute toxicity of **18** determined in the hen's fertile egg screening test is >50 mg/kg. The compound is not cytotoxic.

In summary, thiosemicarbazones of the natural compound madurahydroxylactone represent a new prototype of nonsteroidal inhibitors of the enzyme estrone sulfatase. The most potent inhibitor is not estrogenic and shows low acute toxicity. MHL thiosemicarbazones are therefore good lead compounds in the search for potent nonsteroidal inhibitors.

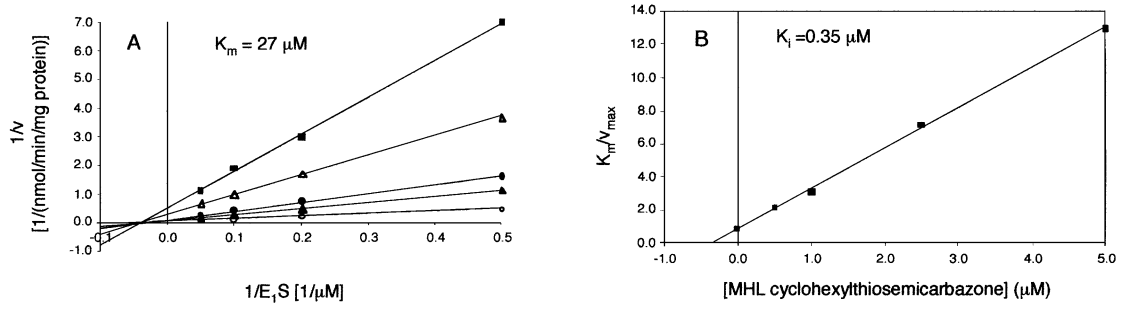

Figure 1. (A) Lineweaver–Burk plot for the inhibition of placental estrone sulfatase activity with MHL cyclohexylthiosemicarbazone **18**. Estrone sulfate (E_1S, 2–20 μM) was incubated with a placental preparation in the presence of **18** [0 (○), 0.5 (▲), 1.0 (●), 2.5 (Δ), or 5 (□) μM] for 20 min at 37 °C in a final incubation volume of 0.5 mL. (B) To obtain apparent K_i for **18**, the slopes (K_m/V_{max}) of the lines from A were plotted as function of the concentration of the inhibitor.

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