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Estrogenic Activity of Musk Fragrances Detected by the E-Screen Assay Using Human MCF-7 Cells

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Abstract. The widespread use of synthetic musk fragrances and the resultant presence of these substances and their metabolites in the aquatic environment (as well as their accumulation in human adipose tissue) raises the question of whether musk fragrances display endocrine and in particular estrogenic activity. A variety of musk fragrances were tested using the E-screen assay. A statistically significant increase in proliferation rate of human MCF-7 breast cancer cells was detected for two nitro musks (musk xylene, musk ketone), a major metabolite of musk xylene (*p*-amino-musk xylene), and the polycyclic musk fragrance AHTN. This indicates that these substances do, in fact, demonstrate estrogenic activity. Coincubation with the antiestrogen tamoxifen showed that the increase in proliferation rate by the musk fragrances is estrogen receptor–mediated. It must be noted, however, that the effective estrogenic strength and estrogenic potency were low compared to 17*b*-estradiol. The naturally occurring fragrance muscone from the group of macrocyclic musk fragrances, a group of substances that have not yet been well characterized in respect to their toxicological properties, has also been shown to be weakly estrogenically active *in vitro*. E-screen analysis showed that the nitro musk metabolites *o*-amino musk xylene and 2-amino-MK, the macrocyclic musk fragrances ethylene brassylate, ethylene dodecandioate, and cyclopentadecanolide, are not estrogenically active.

Synthetic musk fragrances play a large role as additives in cosmetics and detergents (Ford 1998a, 1998b). Data from 1997 show that approximately 8,000 metric tons of musk fragrances are produced yearly. Of these, approximately 70% are polycyclic musk fragrances, 25% are nitro musk fragrances, and about 5% are so-called macrocyclic musk fragrances (Gebauer and Bouter 1997). In 1981 the first indication of residues of these fragrances were detected in the aquatic environment (Yamagishi *et al.* 1981). These substances and/or their metabolites are enriched in tissues of fish and marine animals (Rimkus and Brunn 1996). Human adipose tissue and breast milk have been found to contain musk fragrances (Rimkus *et al.* 1994; Rimkus and Wolf 1996).

The principle means by which nitro musks are broken down is by reduction of the nitro groups. Concentrations of the resulting metabolites in water treatment plants and in surface water have been shown to be 4–40 times as great as those of the original substances (Gatermann *et al.* 1998; Chou and Dietrich 1999). Thus the fragrances of the groups of nitro musk compounds and polycyclic musk compounds are environmentally persistent (Rimkus and Brunn 1996; Pleschka 1999). There is only sparse knowledge about a new group of musk fragrances, the macrocyclic musk compounds (Pleschka 1999). In part, these compounds occur naturally. Muscone, for example, is a component of the musk secretion of the musk deer, and cyclopentadecanolide occurs naturally in the Angelica root *Angelica archangelica* L. (Gebauer and Bouter 1997).

In recent years it has been shown that a large number of anthropogenic substances occurring in the environment display endocrine and in particular estrogenic activity. Estrogenically active substances can have detrimental effects on the reproductive systems of organisms (Guillette *et al.* 1994; Toppari *et al.* 1996). In addition, controversy surrounds the relationship between such xenoestrogens and hormone-induced cancers (Wolff *et al.* 1994; Güttes *et al.* 1998; Høyer *et al.* 1998; Fleming *et al.* 1999). Estrogenic activity has been demonstrated for diverse industrial chemicals as well as for a number of pesticides (Lee and Lee 1996; Toppari *et al.* 1996; Tyler *et al.* 1998). Little is known about potential hormonal and, in particular, estrogenic effects of the musk fragrances (Brunn and Rimkus 1997; Chou and Dietrich 1999). The present study was therefore designed to determine whether musk fragrances and/or their metabolites demonstrate an estrogenic activity and

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whether it is possible to detect such an activity *in vitro*. A total of eight musk fragrances from the three classes of substances listed above as well as three metabolites of the so-called nitro musk fragrances were examined.

Materials and Methods

Reagents

Unless otherwise noted, all reagents and solvents were obtained from Merck (Darmstadt, Germany) or Sigma (Taufkirchen, Germany). Cell culture materials were, unless otherwise noted, obtained from Biochrom (Berlin).

Musk Fragrances

A total of 11 substances were examined, 2 from the group of the nitro musk fragrances, 3 amino metabolites of the nitro musk fragrances, 2 polycyclic musk fragrances, as well as 4 macrocyclic musk fragrances (Figure 1). The nitro musk fragrance, musk xylene (1-*tert*-butyl-3,5-dimethyl-2,4,6-trinitrobenzene, purity 99.5%, MX) and musk ketone (1-(4-*tert*-butyl-2,6-dimetyl-3,5-dinitrophenyl)ethanone, purity 99.5%, MK) were obtained from Promochem (Wesel, Germany). The nitro musk metabolite, *para*-amino-musk xylene (1-amino-4-*tert*-butyl-2,6-dimethyl-3,5-dinitrobenzene, purity > 99.9%, *p*-AMX) and *ortho*-amino-musk xylene (1-amino-2-*tert*-butyl-4,6-dimethyl-3,5-dinitrobenzene, purity 95%, *o*-AMX) were synthesized according to the procedure described by Behechti *et al.* (1998). Musk ketone amine (1-(3-amino-4-*tert*-butyl-2,6-dimetyl-5-nitrophenyl)ethanone, purity > 99.9%, MKA) was synthesized from musk ketone by Wolf-Kižner reduction (Becker *et al.* 1972). The polycyclic musk fragrances HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyrane, purity 98%) and AHTN (1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthalenyl)ethanone, purity 99.0%), as well as the macrocyclic musk fragrances muscone (3-methyl-1-cyclopentadecanon, purity > 99.9%, M), ethylene brassylate (1,4-dioxacycloheptadecane-5,17-dion, purity 95%, EB), ethylene dodecandioate (1,4-dioxacycloheptadecane-5,16-dion, purity > 99.9%, ED) and cyclopentadecanolide (oxacyclohexadecan-2-on, purity > 99.9%, C) were kind gifts of the manufacturers. The structural formulas of the substances tested are shown in Figure 1.

The substances were dissolved in ethanol p.a. The stock solutions contained 10 mmol/L of the test substance, the solutions of *p*-amino musk xylene and musk ketone amine each contained 5 mmol/L.

Test System

The system chosen for detecting potential estrogenic activity was the E-screen assay, developed by Soto *et al.* (1995) and validated by Körner *et al.* (1998). In this system, estrogenic activity is measured using an estrogen receptor–positive human mammary carcinoma cell line (MCF-7). The test depends on determination of an increase in proliferation rate induced by the test substance over the rate measured using a hormone-free control sample. Detection of an increase in proliferation is indirect, taking advantage of the sulforhodamine-B assay (SRB-assay) developed by Skehan *et al.* (1990). In this method the cells are stained, and the extinction is determined photometrically. Cell number is calculated by means of a calibration series.

Measurements of the nitro musk fragrances, their metabolites, the macrocyclic fragrances, and HHCB were performed in 24-well plates (Biochrom). Screening of AHTN was done in 96-well plates (Falcon, Becton-Dickinson, Heidelberg, Germany), according to Körner *et al.* (1999).

Cell Culture

MCF-7 cells were routinely maintained in Dulbecco's modified Eagle's medium (Dulbecco's MEM) with phenol red (= culture medium). For the E-screen assay, cells were cultured in Dulbecco's MEM without phenol red (= experimental medium; Gibco, Karlsruhe, Germany). All culture media contained 5% fetal calf serum (FCS), 100 IU/ml penicillin G (sodium salt), 100 μg/ml streptomycin sulfate, 1.25 μg/ml amphothericin B, and 2 mmol/L glutamine. Dulbecco's MEM without phenol red contained 1% (v/v) [4-(2-hydroxyethyl)-piperazino]-ethane sulfonic acid (HEPES) buffer per liter. The FCS used in the experimental medium was stripped with an active charcoal–dextran mixture according to the method described by Stanley *et al.* (1977) to remove steroids from the serum.

Cells were cultivated in an incubator at 37°C, 5% CO_2, and 95% relative humidity.

Before beginning the assays, the stock solutions were diluted with experimental medium. To avoid solvent concentrations greater than 0.1% (v/v), the maximum test substance concentration measured was 10 μmol/L, except for *p*-amino musk xylene and musk ketone amine (5 μmol/L)

E-Screen

The following specifications apply to screening with 24-well plates. AHTN was screened using 96-well plates, and the specifications for these measurements are shown in brackets.

Day 0: Passage and seeding of the cells in culture medium 24-well (96-well) plates at 10,000 (1,500) cells per well.

Day 1: Aspiration of the culture medium. Addition of experimental medium containing the substance to be tested (see Musk fragrances).

The assay was begun by addition of the following to each plate: A hormone-free negative control [4 (8) wells each], one plate as positive control (17*b*-estradiol at a concentration of 10^{-9}–10^{-13} mol/L (10^{-9}–10^{-12} mol/L), 4 (8) wells each, as well as the test substances at 5 (7) different concentrations, also 4 (8) wells each.

Tag 6: The assay was stopped by removal of the experimental medium and fixation of the cells.

Coincubation

To test for receptor mediation of the increase in proliferation rate, cells were coincubated with 1 μmol/L of the antiestrogen tamoxifen. The experimental conditions of coincubation were the same as those used in the normal E-screen tests.

Sulforhodamine B–Assay

After aspiration of the experimental medium, the cells were fixed in 10% trichloroacetic acid for 30 min at 4°C, rinsed with tap water, and allowed to dry for about 2 h at 40°C max. Cells in the 24-well plates were then stained with 250 μl of a 0.4% sulforhodamine B solution (dissolved in 1% acetic acid) per well. For the 96-well plates, 50 μl per well were used. After 10 min incubation at room temperature the cells

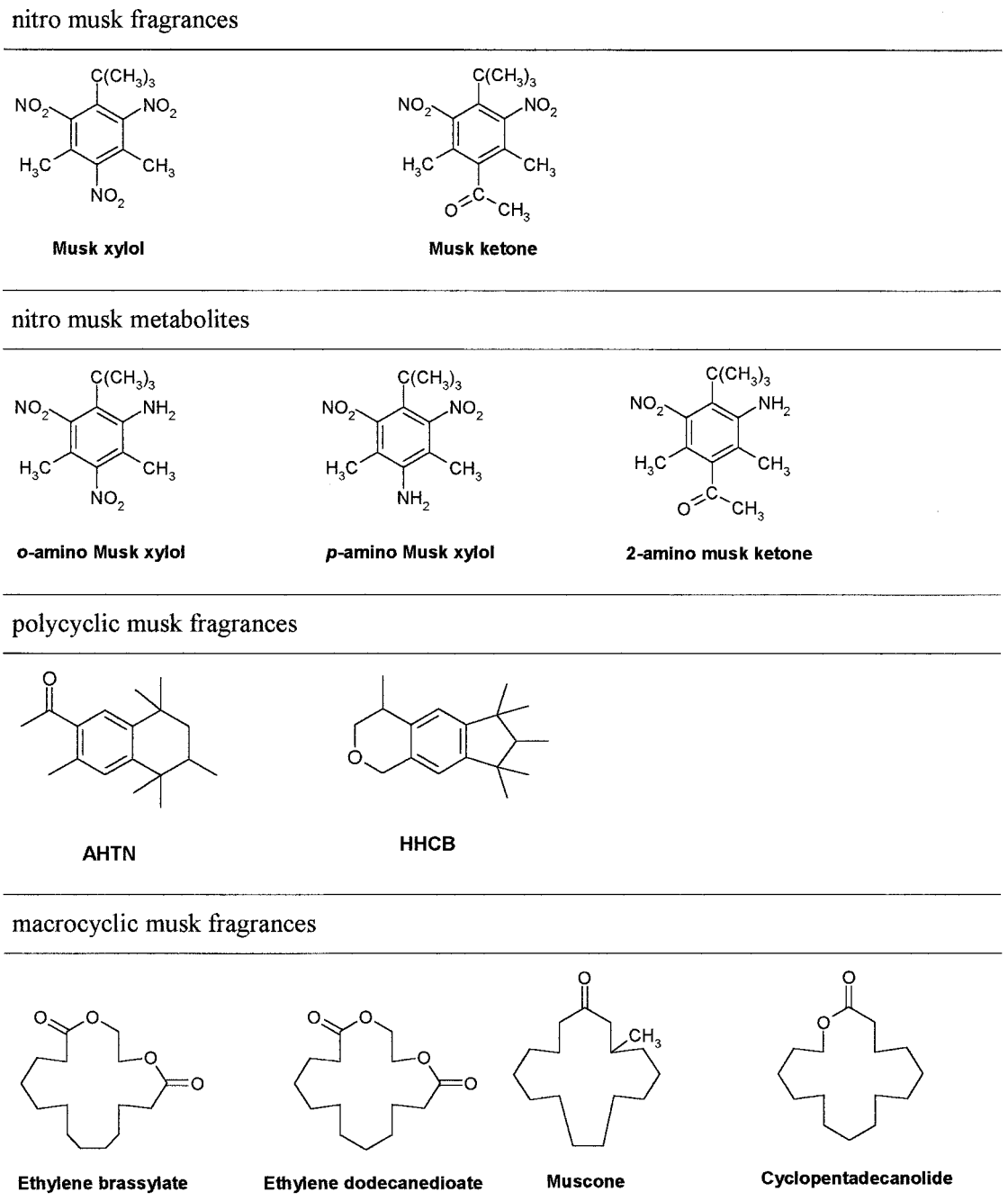

Fig. 1. Musk fragrances and their metabolites analyzed using the E-screen assay

were washed with 1% acetic acid and again dried at 40°C. The dye was then removed from the cells with 500 μl (100 μl) 0.01 M TRIS buffer at pH 10.5. For the 96-well plates, this last step was omitted and the plates were measured directly in the photometer.

Photometric determination of the total protein content was performed at a wavelength of 550 nm. For technical reasons, a reference wavelength of 620 nm was used rather than 630 nm, as used by Körner *et al.* (1998). For AHTN, extinction measurements a reference wavelength of 630 nm was used.

Conversion of extinction value to cell number was made using a calibration series in which the extinction of increasing volumes of defined cell numbers was measured.

Evaluation of the Assays

The assay method depends on an increase in proliferation rate over that of a hormone-free control sample. Determination of efficacy compared to 17*b*-estradiol (E2) was calculated according to the literature (Soto *et al.* 1995; Körner *et al.* 1998). The following parameters were calculated.

Proliferative Effect (PE): The PE is the ratio of the largest mean cell number that was obtained with the test substance or E2 to that of the negative control:

$$PE = \frac{\text{cell number (test substance, or E2)}}{\text{cell number (negative control)}}.$$

The negative control therefore always has a PE of 1.

Because this is a biological system in which the distribution does not follow a Gaussian bell-shaped curve but is right skewed, the geometric mean rather than the arithmetic mean was obtained from the calculated cell number.

The PE value was obtained using the following formula:

$$PE = 10^{\overline{\log x} - \overline{\log y}} \text{ with}$$

$\overline{\log x}$ = arithmetic mean of the decadic logarithm of the cell number with the test substance or E2, and $\overline{\log y}$ = arithmetic mean of the decadic logarithm of the cell number with the negative control.

Relative Proliferative Effect (RPE): The RPE is a comparison of the maximal proliferation with the test substance to that of E2. The RPE indicates whether a substance is a stronger or weaker agonist to the estrogen receptor. For the sake of clarity it is expressed in percent.

$$RPE(\%) = \frac{PE - 1 \text{ (test substance)}}{PE - 1 \text{ (E2)}} \times 100,$$

whereby the 1 is derived from the negative control (see above).

Relative Proliferative Potency (RPP): The RPP value describes the ratio of concentrations of 17*b*-estradiol and of the test substance that were necessary to obtain maximum induction of the cell numbers (EC_{100}) (Soto *et al.* 1995). Thus the RPP provides an indication of the potency and therefore the efficacy of a substance relative to 17*b*-estradiol. The RPP value is calculated as follows.

$$RPP = \frac{EC_{100}(E2)}{EC_{100}(\text{test substance})}$$

This value has no dimension.

Statistical Analysis

The data were evaluated using the statistical software package BMDP/Dynamic, release 7.0 (Dixon 1993). The t values used were calculated using Microsoft EXCEL 97.

Variance in cell number between repeat measurements of the negative control within one plate was estimated using one-way analysis of variance (ANOVA, BMDP). Cell numbers were transformed logarithmically because their distribution is skewed to the right with only positive values. The estimates and the t values were used in a formula to calculate a one-sided upper limit for the random deviation of PE values. To this end, the following equation was used, based on the law of error-propagation and the assumption that the variance of the negative controls and of the test-substance measurements were the same:

$$UL_{lg(PE)} = t_{p;DF} * SD_{lg(PE)}$$

UL = one-sided upper limit of the range of random dispersion
lg(PE) = decadic logarithm of the PE value
t = t value from the t distribution (t table)
p = statistical confidence, 99.9%
DF = degrees of freedom from ANOVA, 191 (calculated from the number of 255 measurements −64 negative controls)
$SD_{lg(PE)}$ = standard deviation of the logarithm of the PE value, 0.033

Using this equation, the following limits for varying deviation were calculated:

For error probability $\alpha = (1 - p) < 0.001$: $PE > 1.27$
very distinct (relevant)

For error probability $\alpha = (1 - p) < 0.005$: $PE > 1.22$
distinct (relevant)

A PE value of less than 1.2 was considered not significantly different from 1.

Statistical Evaluation of the Results Obtained for AHTN

As the test setup for AHTN varied slightly from the setup for musk fragrances (see the section on E-screen), the statistical evaluation of this substance was performed separately. The Komolgorow-Smirnoff test for normal distribution was performed (Sigmastat, version 2.0 for Windows 95). Using EXCEL 97 for Windows 95 an F-test for homogeneity of variance as well as a *t* test for significant differences of mean values were carried out.

Results

Figures 2 and 3 present the change in rate of proliferation (PE value) for the musk fragrances relative to the solvent controls (0.1% EtOH). The results are presented as the geometric mean and the dispersion factor of individual geometric means of the data points. The results show that musk xylene, musk ketone, and *para*-amino musk xylene increase the rate of proliferation with a statistical confidence of 99.9% ($PE > 1.27$). Coincubation with 1 μmol/L tamoxifen eradicated this effect, indicating that the increase observed is the result of a receptor-mediated estrogenic effect (Figure 2).

No increase in proliferation could be detected for *o*-amino-musk xylene or musk ketone. The value for musk ketone lies within the range of deviation obtained for the control samples. The results for *ortho*-amino musk ketone indicate that this substance is apparently cytotoxic at a concentration of 10 μM in the test system used.

From Figure 3 it is apparent that a statistically significant increase in proliferation was only found with muscone and AHTN. The *t* test for AHTN showed a significant difference over the negative control at a concentration of 10^{-5} mol/L ($p < 0.001$). For muscone the PE value was greater than 1.22. The increase in proliferation for muscone is therefore distinct ($p < 0.005$). Just as for the nitro musk fragrances (Figure 2), coincubation with 1 μmol/L tamoxifen showed that the estrogenic activity was receptor-mediated.

Increase in proliferation of HHCB was above the required level of significance of > 1%. This indicates that no estrogenic activity was detectable for HHCB. Coincubation with tamoxifen was therefore not performed. Table 1 shows the mean PE values of the various substances tested here, the mean PE, RPE, and RPP values. These results demonstrate that all of the musk fragrances examined are only weak agonists of the estrogen receptor. The estrogenic potency (RPP) is five to six orders of magnitude weaker than for E2.

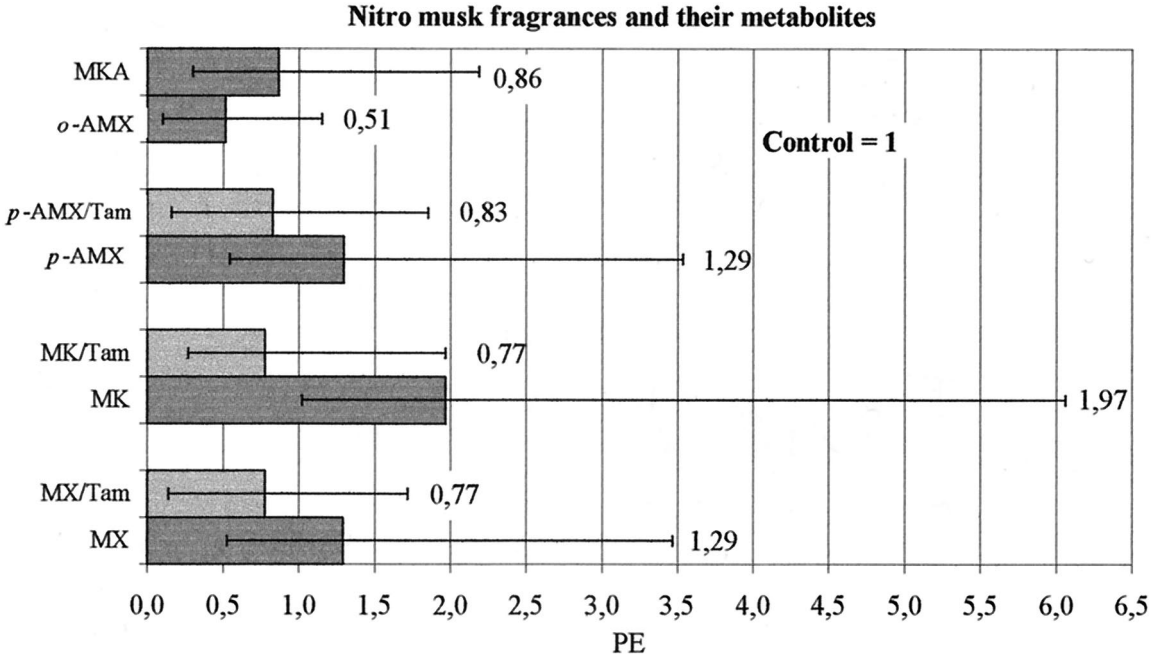

Fig. 2. PE values of nitro musk fragrances and their metabolites, presented as geometric means and dispersion factor. The concentration of the test substances was 10 μM with the exception of *p*-AMX and MKA (5 μM). Samples were coincubated with 1 μM tamoxifen (Tam). Three assays each (n = 3) were carried out for MK, MKA, MX/Tam, MK/Tam, and *p*-AMX/Tam. For MX and *o*-AMX, n = 4. For *p*-AMX, n = 6

Discussion

A total of eight musk fragrances, two from the group of nitro musks, three amino metabolites of the nitro musks, two polycyclic, and four macrocyclic musk fragrances were tested for estrogenic activity by E-screen assay. The nitro-musk fragrances musk xylene and musk ketone could be shown to possess estrogenic activity *in vitro* (Figure 2). Similar results were previously obtained for musk xylene (Schuller 1998).

Musk ketone has not previously been tested by E-screen analysis. In the test system used in this study, this substance showed an affinity to the estrogen receptor three times greater than musk xylene (Table 1).

Reduction to the amine is the first step in the transformation of nitro fragrances (Gatermann *et al.* 1998; Rimkus *et al.* 1999). When musk ketone is reduced to the amine, the estrogenic activity is lost. Reduction of the nitro group of musk xylene, in contrast, leads to an increase in estrogenic potency (Figure 2). *Para*-amino musk xylene is the main metabolite of musk xylene in the aquatic environment (Rimkus *et al.* 1999). Reduction to the amine in the para position leads to the same proliferative effect at half the concentration, 5 μmol/L, as 10 μmol/L of the native substance, musk xylene (Figure 2).

The RPE values are nearly equal at 12.7% and 11.4% for *p*-AMX and MX, respectively (Table 1). In contrast, reduction of the amino group in the ortho position results in a clearly cytotoxic effect (Figure 2). The PE value drops with increasing concentration, always having a value of less than 1 (data not shown). Reduction of a nitro group of the aromatic ring system of musk fragrances appears to result in a position-dependent variable influence on the biological effect of the respective metabolites

The group of Chou and Dietrich (1999) was not able to demonstrate binding of musk xylene and musk ketone to the estrogen receptor of *Oncorhynchus mykiss* or *Xenopus laevis*. The metabolites, *para*-amino-musk xylene, *ortho*-amino-musk xylene, and 2-amino-MK, however, demonstrated estrogen receptor binding in both species. The discrepancy of these results with those of the present study may be a result of the different test system but may also lie in the differences in binding of estrogen receptors of fish, frogs and humans (Petit *et al.* 1995). Of the musk fragrances that are presently most commonly used, the polyclic musks (Barbetta *et al.* 1988; Gebauer and Bouter 1997) only AHTN was seen to be estrogenically active (Figure 3). With an RPE value of 15.1% AHTN is a partial agonist of the estrogen receptor (Table 1). Its estrogenic potency, in comparison to 17*b*-estradiol, is weak, however (Table 1).

In the E-screen, HHCB only showed a statistically insignificant increase in proliferation. The PE value of 1.17 (Figure 3) was below the statistically established limit of 1.2 and therefore within the range of deviation of the negative control. In the E-screen, therefore, HHCB must be considered estrogenically inactive.

Other authors have reported weak estrogenic activity for HHCB. Schuller (1998) tested Galaxolide® in the E-screen for estrogenic activity. Galaxolide, however, is a commercial mixture of HHCB and plasticizers, containing as much as 50% of the latter substances, including diethylhexylphthalate (DEHP). DEHP itself is negative in the E-screen assay (Jobling *et al.* 1995; Harries *et al.* 1997; Schuller 1998). The mixture Galaxolide was shown to have an RPE value of about 36%. The author, however, notes that the data points showed a large standard deviation. He was unable to

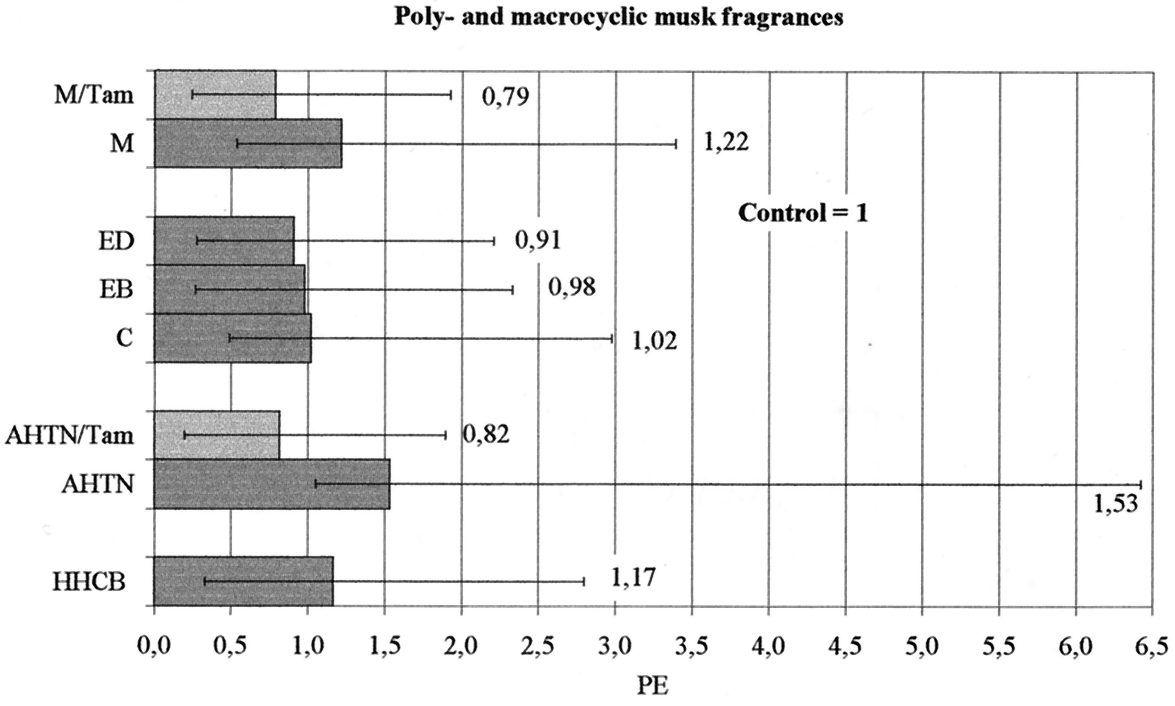

Fig. 3. PE values of macro- and polycyclic musk fragrances presented as geometric means and dispersion factors. The concentration of the test substances was 10 μM. Samples were coincubated with 1 μM tamoxifen (Tam). The concentration of AHTN was 5 μM during coincubation. Three assays each (n = 3) were carried out for M/Tam. For AHTN, AHTN/Tam, C, EB, and ED n = 4. For M n = 5, and for HHCB, n = 8

Table 1. PE, RPE, and RPP values of the musk fragrances tested positive in the E-screen assay

Substance	PE Value	PE E2	RPE (%)	RPP ($\times 10^{-5}$)
Musk xylol	1.29	3.55	11.4	3.3
Musk ketone	1.97	4.46	28.0	7
p-AMX	1.29	3.29	12.7	5
AHTN	1.53	4.51	15.1	1
Muscone	1.22	3.69	8.2	4.6

reach an unequivocal conclusion as to whether the results were caused by a possible synergistic reaction of HHCB and DEHP (Schuller 1998), as pure HHCB, tested in the present study using the same test system, was classified as estrogenically inactive.

The group of Seinen *et al.* (1999) tested HHCB for estrogenic activity both *in vitro* and *in vivo. In vitro* ERα-transfected human HEK293 cells showed a weak estrogenic activity. In this test, the potency was shown to be only 15% of that of 17*b*-estradiol (100%) (Seinen *et al.* 1999). *In vivo*, in a mouse uterus test, no estrogenic activity was detectable (Seinen *et al.* 1999).

The macrocyclic musk fragrances, which in part occur naturally (Gebauer and Bouter 1997), were not previously tested for endocrine activity. The macrocyclic substances tested in the present study were estrogenically inactive, with the exception of muscone, which showed a weak, receptor-mediated estrogenic activity (Figure 3).

Conclusions

By means of the E-screen assay, a number of musk fragrances as well as *p*-amino musk xylene were shown *in vitro* to possess estrogenic activity. It must be noted, however, that the potency of all the substances tested was low. The RPE values were all less than 30% (Table 1). The musk fragrances found here to be estrogenically active should therefore be classified as partial agonists. In relation to other substances such as the phytoestrogens (Schuller 1998; Bitsch *et al.* 2001) the estrogenic potency of the musk fragrances compared to 17*b*-estradiol is low.

Nitro musk fragrances and polycyclic musk compounds become enriched in the aquatic environment (Rimkus and Brunn 1996). The extent to which a potential accumulation of even weakly endocrinic substances may endanger the aquatic environment, in particular, still remains an open question. Also unknown is the question of potential dangers to humans through the use of these substances in cosmetics because nitro musk fragrances and polyclic musk fragrances have both been shown to be lipophilic in the body and become concentrated in human adipose tissue and in human breast milk (Rimkus *et al.* 1994; Rimkus and Wolf 1996).

At present, macrocyclic musk fragrances are used only sparingly by the perfume industry (Gebauer and Bouter 1997). Although they are biologically more readily catabolized, which represents an advantage over the nitro musk fragrances and the polycyclic compounds, they have the disadvantage of being much more expensive than the polycyclic or nitro musks (Gebauer and Bouter 1997). In the E-screen assay, ethylene brassylate, ethylene dodecandioate, and cyclopentadecanolide fail

to show any affinity to the estrogen receptor. Only muscone was estrogenically weakly active (Table 1). Its RPE value, however, was just below 10%. Below this value, the estrogenic potency can be considered negligible (Sonnenschein and Soto 1998). With regard to their estrogenic activity, macrocyclic musk fragrances would offer an alternative to the nitro- and polycyclic-musk compounds used presently. Extensive *in vivo* and in particular dose-dependent studies will provided indications as to the biological relevance of the in vitro results presented here.

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