28.83, 28.66, 11.57; MS, m/e 330 (M^+, 2), 312 ($M - H_2O$, 30), 295 (100), 264, 216, 203.

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Registry No. (+)-**4**, 59372-72-4; **5S**, 96844-07-4; **6**, 1120-73-6; **7**, 111954-77-9; **8**, 111954-78-0; **9**, 111849-24-2; **10**, 111849-21-9; **11**, 111849-22-0; **11** (ethylene ketal), 111849-18-4; **12**, 111849-23-1; **12** (ethylene ketal), 111954-74-6; **13**, 111849-17-3; (±)-**15**, 111849-26-4; (±)-**16**, 110455-66-8; **17**, 111849-25-3; (±)-**19**, 111849-27-5; (±)-**20**, 111849-28-6; *cis*-**21**, 111849-11-7; *trans*-**21**, 111849-12-8; *cis*-**21** (sulfoxide), 111849-13-9; *trans*-**21** (sulfoxide), 111849-14-0; **22**, 111849-15-1; **23**, 111849-16-2; **24**, 96759-78-3; *cis*-**25**, 111849-19-5; *trans*-**25**, 111954-75-7; **26**, 96759-77-2; **27**, 96759-79-4; **28**, 111954-76-8; **29**, 96759-80-7; **30**, 111849-20-8; **31**, 96759-82-9; **32**, 96759-83-0; **33**, 96759-84-1; **34**, 96759-85-2; **35**, 96844-05-2; (±)-(*R**,*R**)-**36**, 111849-08-2; (±)-(*R**,*S**)-**36**, 111849-07-1; (±)-(*R**,*R**)-**37**, 111849-10-6; (±)-(*R**,*S**)-**37**, 111849-09-3; **38**, 1120-80-5; (±)-(*R**,*R**)-**39**, 111849-01-5; (±)-(*R**,*S**)-**39**, 111849-02-6; **40**, 111849-03-7; (±)-(*R**,*R**)-**41**, 111849-04-8; (±)-(*R**,*S**)-**41**, 111849-05-9; **42**, 111849-06-0; **43**, 64493-28-3; (+)-(*S*)-$CF_3C(OMe)(Ph)COCl$, 20445-33-4; $HOCH_2C(CH_3)_2CH_2OH$, 126-30-7; $BrCH_2C(CH_3)_2CH_2OCOCH_3$, 3492-41-9; $BrCH_2C(CH_3)_2CH_2OH$, 40894-00-6; $Ph_3PMe^+Br^-$, 1779-49-3; *i*-$PrSO_2Cl$, 10147-37-2; 1-methyl-1-cyclopentene, 693-89-0; 2-cyclopentenone, 930-30-3.

Microbial Transformation of Zearalenone. 2. Reduction, Hydroxylation, and Methylation Products

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Microbial transformations have been employed as a means of preparing analogues of the resorcylic acid lactone zearalenone. Microbial transformation products were initially identified by thin-layer chromatography of fermentation extracts and then prepared by large-scale incubations. Each metabolite was subjected to structural elucidation employing carbon-13 and proton NMR, mass spectrometry, and infrared analysis. Metabolites were identified as α- and β-zearalenol, α- and β-zearalanol, zearalanone, 8′(*S*)-hydroxyzearalenone, 2,4-dimethoxyzearalenone, and 2-methoxyzearalenone. Binding affinities to rat uterine estrogen receptors were carried out. Only those metabolites having a free 4-phenolic group were capable of binding to the estrogen receptor. However, 8′-hydroxyzearalenone, even with a 4-phenolic hydroxyl, did not bind to the receptor. It is possible that hydrogen bonding of the aliphatic hydroxyl groups to the C-6′ carbonyl of zearalenone or equilibrium between the hydroxy ketone and its tautomeric hemiketal may lead to distortion of the conformation of the molecule resulting in loss of binding to the receptor.

Introduction

Zearalenone 1, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone, is a fungal metabolite produced by certain *Fusarium* sp. when hosted on corn and other cereal grains. This compound possesses estrogenic- and growth-promoting activity in laboratory and farm animals.[1,2] The economic loss associated with impaired fertility in cows[3] and hyperestrogenism in swine has promoted further investigations on the metabolism of zearalenone by mammalian species[4-8] to determine the structure of the active compound(s).

In view of the fact that zearalenone is a good growth promoter in farm animals, and since diethylstilbestrol was found to be potentially carcinogenic[9] and therefore banned as a growth promoter in animals, several investigators were led to conduct a systematic study on the biological activities of zearalenone and several of its synthetic analogues[10-12] and to establish structure–activity relationships

Table I. Percent Microbial Transformation of Zearalenone to α- and β-Zearalenols

microorganism	α-zearalenol (%)	β-zearalenol (%)
Absidia coerulea MR-27B	*a*	5
Absidia spinosa NRRL 3033	4	*a*
Aspergillus niger ATCC 11394Y	12	4
Fusarium avenaceum 12 F-6	5	6
Fusarium oxysporum 5F-3	5	5
Mucor bainieri NRRL 2988	*a*	60
Penicillium stipitatum MR-336	8	8
Streptomyces griseus ATCC 13273	40	7
Streptomyces rimosus NRRL 2234	12	*a*
Streptomyces rutgersensis NRRL B1256	8	23

[a] No transformation.

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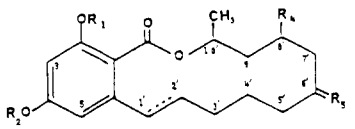

	Compound	R_1	R_2	R_3	R_4	R_5	C-1'-C-2'
1	Zearalenone	H	H	H	H	O	HC=CH
2	α-Zearalenol	H	H	H	H	H, OH	HC=CH
3	β-Zearalenol	H	H	H	H	OH, H	HC=CH
4	α-Zearalanol	H	H	H	H	H, OH	CH_2CH_2
5	β-Zearalanol	H	H	H	H	OH, H	CH_2CH_2
6	Zearalanone	H	H	H	H	O	CH_2CH_2
7	8'(s)-Hydroxyzearalenone	H	H	H	OH	O	HC=CH
8	2,4-Dimethoxyzearalenone	CH_3	CH_3	H	H	O	HC=CH
9	2-Methoxyzearalenone	CH_3	H	H	H	O	HC=CH
	Zearalenone-4-O-β-glucoside*	H	glucose	H	H	O	HC=CH

* See Ref. 14.

Figure 1. Structures of zearalenone and its microbial metabolites.

for this series. Microbial transformations have been extensively used in the preparation of analogues of zearalenone, whose preparation would have been difficult by other routes. Their structural elucidation using spectroscopic techniques and a description of their biological activities is the subject of this article.

Results and Discussion

The outstanding successes realized in microbial transformations of steroids and of other terpenes[13] indicated that a similar approach with zearalenone could provide new and potentially useful analogues. A broad program of screening microorganisms for their abilities to achieve chemical transformations of zearalenone was undertaken. Some 170 cultures were examined, and numerous of these provided different metabolites of zearalenone, some in almost quantitative yield (HPLC). The types of chemical transformations observed include reduction of the carbonyl ketone, reduction of the double bond, hydroxylations, O-methylation, O-glucosidation,[14] and cleavage of the lactone.[15] Several of the best metabolite-accumulating cultures were selected for preparative scale work.

Streptomyces griseus (ATCC 13273) and *Mucor bainieri* (NRRL 2988) were used to produce α-zearalenol (**2**) and β-zearalenol (**3**) in 40% and 60% yields, respectively. The identity of the products was established by comparison of the IR and ^1H NMR spectra and melting points with those reported in the literature[16] as well as those obtained by using authentic samples.[17] Table I shows that most microorganisms, except for *M. bainieri* and *S. rimosus*, produced a mixture of both isomers. These observations are quite interesting in view of the stereospecificity of enzymatic reactions. Thus, because of the relative flexibility of the lactone ring, it is quite possible that a single enzyme may catalyze the reduction of the C6'-ketone, resulting in the formation of the isomeric C6'-diols. However, it is quite possible that two separate reductases may be involved. Experiments designed to use cell-free systems in the presence of 4-*pro*-*S*[^3H]NADPH and 4-*pro*-*R*[^3H]-NADPH should be helpful in explaining this observation.[18]

Aspergillus ochraceous (NRRL 405) and *Aspergillus niger* (X-170) gave 20% and 50% yields of α-zearalanol (**4**) and β-zearalanol (**5**), respectively. The spectral data for these two compounds were essentially similar to those obtained for compounds **2** and **3** except for the signals for the C-1'–C-2' double bond. The ^1H NMR spectrum lacks the olefinic proton signals but has additional new signals at δ 3.58 and 2.10 (**4**) and at δ 3.50 and 2.15 (**5**). Mass spectral data showed a molecular ion at *m/e* 322.

Saccharomyces cerevisiae (NRRL Y2034) was used to produce zearalanone (**6**) in 20% yield. The IR spectrum and mass spectral (*m/e* 320, 2 mass units more than zearalenone) behavior supported the structure as **6**. The loss of the olefinic proton signals at the C-1' and C-2' positions was evident in the ^1H NMR spectrum. The ^{13}C NMR spectrum of **6** showed the loss of two signals at 135.24 and 132.54 ppm and the appearance of two new signals at 37.83 and 32.08 ppm, consistent with the saturation of the olefinic double bond at C1'–C2'. It is interesting to note that only this microbe produced this metabolite. Most microbes saturating the olefinic double bond also showed reduction of the C-6' carbonyl.

Streptomyces rimosus (NRRL 2234) was used to produce 8'(*S*)-hydroxyzearalenone (**7**) in 32% yield. The mass spectrum showed an ion at *m/e* 334, indicating the presence of one additional oxygen in zearalenone. The ^1H NMR spectrum (3.71 ppm, ^1H) and ^{13}C NMR spectrum (64.6 ppm) indicate that hydroxylation had occurred at one of three possible methylene carbon atoms. The assignment of the hydroxyl group to the C-8' position is essentially in agreement with the results obtained by Mirocha's group[19,20] and Bolliger and Tamm,[21] using 8'-hydroxyzearalenone produced by *F. roseum* in which the C-10' and C-7' protons were shifted downfield by 0.76 and 0.25 ppm, respectively. The ^{13}C NMR spectrum showed that the signal for the C-7' carbon was deshielded (14.7 ppm) more than the C-9' carbon (4.3 ppm) which is attributed to its proximity to the C-6' carbon carbonyl. The stereochemistry of the hydroxylation was based on comparison to an authentic sample of 8'(*S*)-hydroxyzearalenone in which X-ray crystallographic data showed that the hydroxy group was in the 8'*S* configuration.[19,22]

Cunninghamella bainieri (ATCC 9244B) produced two major metabolites in 25% and 22% yields that were identified as zearalenone derivatives **8** and **9**, respectively. Both compounds showed IR spectra with no absorption bands at 3300 cm^{-1}, indicating the disappearance and interruption of the intramolecular hydrogen bonding between the C-2 hydroxyl group and the lactone carbonyl that was shifted downfield (37 cm^{-1}) in comparison to zearalenone. The ^1H NMR (methyl group absorption at 3.8), ^{13}C NMR (absorbances at 55.4 ppm), and mass spectral properties of these two compounds are consistent

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Table II. Estrogen Receptor Affinity of Zearalenone Metabolites

compound	RBA[a]
estradiol	100.0
zearalenone	2.0
zearalanone	2.0
α-zearalenol	1.9
β-zearalenol	1.9
α-zearalanol	15.8
β-zearalanol	1.3
8′(*S*)-hydroxyzearalenone	0.0
2-methoxyzearalenone	2.0
2,4-dimethoxyzearalenone	0.0
zearalenone-4-*O*-β-glucoside	0.0

[a] RBA = Relative binding affinity for the rat uterine estrogen receptor. See Experimental Section for definition and determination.

with the formation of a mono- and di-O-methylation of the parent compound. Thus, compound 8 was found to be 2,4-dimethoxyzearalenone. With respect to the monomethoxylated derivative (9), the assignment of the methyl group to the C2-OH group was based on both the IR and ^{13}C NMR spectra. The IR spectrum showed no absorption at 3300 cm^{-1}, while the frequency at 1688 cm^{-1} which is assigned to the lactone carbonyl in zearalenone was shifted to 1725 cm^{-1} in 9. These results indicate the disappearance and interruption of the intramolecular hydrogen bonding between C2-OH and the lactone carbonyl. The ^{13}C NMR spectra showed that substitution at the C-2 phenolic group had a significant effect on the aromatic ring system. Thus, the intramolecular hydrogen bonding at C-2 causes the lactone carbonyl to remain coplanar with the aromatic ring resulting in increased electron density around C-6. In compounds that do not have a free C2-OH group, the C-6 carbon resonance was found to be shielded by about 7.2 ppm. Thus, substitution at C2-OH forces the lactone carbonyl out of the plane of the aromatic ring system and causes increased polarization of the bond between C-1 and C-12′.

Earlier studies in our laboratories showed that *Thamnidium elegans* and *M. bainieri* transformed zearalenone to zearalenone-4-*O*-β-glucoside[14] while *Gliocladium roseum* transformed zearalenone to a racemic mixture of 1-(3,5-dihydroxyphenyl)-10′-hydroxy-1-undecen-6′-one and 1-(3,5-dihydroxyphenyl)-6′-hydroxy-1-undecen-10′-one.[15]

Biochemical Studies. The binding affinities of the zearalenone metabolites for the estrogen receptor were measured by a competitive binding assay with 17β-[^3H]-estradiol. Rat uterine cytosol was used as receptor source and the dextran coated charcoal method (DCC) was applied.[23] The relative binding affinities (RBA) are given as the ratio of the molar concentrations of 17β-estradiol and zearalenone required to decrease the receptor-bound radioactivity by 50%, multiplied by 100. The semilogarithmic plot of bound radioactivity vs molar concentrations of the zearalenone derivatives exhibited curves parallel to those of 17β-estradiol, suggesting a common binding site for all of the compounds that showed competition.

Table II shows the RBA for the zearalenone compounds isolated by microbial transformations. Zearalenols and zearalanols were found to have a low binding affinity for the estrogen receptor, with α-zearalanol showing the highest activity (RBA = 15.8%). A three-dimensional structure of α-zearalanol shows a relatively close spatial similarity to that of estradiol in which both are about 10–11 Å in length and are of similar lipophilicity.[24] These studies show the importance of a free 4-phenolic group for binding to the estrogen receptor. Thus, those metabolites that have a blocked 4-phenolic group by either methylation or glycosylation[14] were found to be biologically inactive. Compounds with a 6′-ketone or 6′-hydroxyl group were found to compete with estradiol, indicating the need for an electron-rich center around the C-6′ position. It is interesting to note that compound 7 showed no binding to the estrogen receptor, which may be explained by the fact that the 8′-OH can form hydrogen bonding with the C-6′ ketone or may be due to equilibration between the hydroxy ketone and tautomeric hemiketal. Thus, the loss of activity may be attributed to the change in the conformation of the molecule resulting in loss of binding to the estrogen receptor.

Experimental Section

General. Melting points were determined on a Fisher-Jones hot plate apparatus and are uncorrected. Infrared spectra were taken with a Nicolet 5D XC FT-IR spectrophotometer using KBr or Nujol disks. Mass spectra were determined on an LKB 9000 GC mass spectrometer. ^1H NMR and ^{13}C NMR spectra were obtained on a 300-MHz Nicolet NT-300-W3 spectrometer using tetramethylsilane (TMS) as the internal standard. Zearalenone and other authentic zearalenone standards were provided by ICM, Terre Haute, IN. Authentic sample of 8(*S*)′-hydroxyzearalenone was obtained from Dr. Chester Mirocha, University of Minnesota.

Cultures and Fermentation Screening Procedures. Fungal stock cultures were stored at 4 °C and maintained on Saboraud-dextrose agar (Difco) slants.

Biotransformation experiments were performed by using shake culture techniques by a two-stage fermentation procedure in a medium consisting of 2% glucose, 0.5% yeast extract, 0.5% K_2HPO_4, 0.5% neopeptone, and 0.5% NaCl. The medium was adjusted to pH 7 before sterilization by autoclaving at 121 °C and 15 lb pressure for 15 min. After 72 h of incubation in the above medium, 5 mL of stage I culture was used as the inoculum for fresh stage II cultures (50 mL/250-mL flask). After 24 h of incubation of stage II cultures, zearalenone was added (0.25 mg/mL medium) as a suspension in dimethylformamide (180 mg/mL, DMF). Culture controls consisted of fermentation blanks in which the organism was grown under identical conditions without substrate. Substrate controls consisted of sterile media containing the same amount of substrate and incubated under the same conditions. The fermentations were sampled by extraction of 5 mL of culture broth with 5 mL of chloroform. After evaporation of the solvent, the residue was spotted on precoated silica gel TLC plates (0.25 mm) that were developed in chloroform–acetone (8:2), normal phase, or acetonitrile–ethanol–15% NaCl, reversed phase, and visualized by spraying with 1% ceric sulfate in 3N H_2SO_4. All preparative scale incubations were conducted with 500 mg of zearalenone and with protocols established on smaller scale screening experiments. Most transformations were continued until no further increase in metabolite(s) was formed (usually 3–4 days). Both cells and the fermentation browth were extracted separately (3×) with a mixture of chloroform–methanol (9:1, v/v). The combined chloroform extracts were washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under vacuo.

Isolation and Purification. Both preparative TLC and column chromatography were used for isolation of metabolites. Preparative thin-layer chromatography was carried out on 1-mm-thick silica gel GF precoated plates that were developed twice in chloroform–methanol (97:3, v/v). Column chromatography was carried out by using silica gel columns that were eluted with a gradient of a chloroform–methanol mixture of increasing polarity.

Zearalenone. Zearalenone (1) was provided by ICM and possessed physical properties identical with those reported in the literature, including ^1H NMR ($CDCl_3$): δ 1.36 (3 H, d, C-11′), 1.67 (2 H, m, C-8′), 1.81 (2 H, m, C-4′), 2.10 (2 H, m, C-3′), 2.31 (2 H,

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m, C-9′), 2.60 (2 H, m, C-7′), 2.90 (2 H, m, C-5′), 5.01 (1 H, m, C-10′), 5.88 (1 H, m, C-2′, J = 15.5 Hz), 6.40 (1 H, d, C-5, J = 2.5 Hz), 6.44 (1 H, d, C-3, J = 2.5 Hz), and 7.01 (1 H, d, C-1′, J = 15.5 Hz). ^{13}C NMR ($CDCl_3$): δ 21.07 (C-11′), 21.09 (C-8′), 22.39 (C-4′), 31.08 (C-3′), 34.79 (C-9′), 36.76 (C-7′), 43.03 (C-5′), 74.52 (C-10′), 102.96 (C-3), 103.90 (C-1), 108.96 (C-5), 132.54 (C-2′), 135.24 (C-1′), 144.05 (C-6), 160.05 (C-4), 165.46 (C-2), 171.37 (C-12′), and 211.82 (C-6′).

α-Zearalenol (2) was produced by *Streptomyces griseus* ATCC 13273 in 40% yield and other microorganisms in lower yield (Table I): mp (from $CHCl_3$–hexane) 169–170 °C [lit.[17] mp 168–169 °C], R_f 0.62, t_R 11 min. IR (KBr): 3500, 3180, 1630, 1610, and 968 cm^{-1}. Mass spectrum (direct probe): m/e 320 (M^+), 302, 281, 233, 188 (100%), 179, 125, 112, and 97. The TMS derivative showed M^+ m/e 536, 464, 446 (100%), 320, 305, 260, 197, and 169. ^{1}H NMR ($CDCl_3$): δ 1.49 (3 H, d, C-11′), 1.51 (2 H, m, C-8′), 1.73 (2 H, m, C-4′), 1.85 (2 H, m, C-3′), 1.95 (2 H, m, C-9′), 2.42 (2 H, m, C-7′), 2.55 (1 H, m, C-5′), 3.85 (2 H, m, C-6′), 5.08 (1 H, m, C-10′), 5.89 (1 H, m, C-2′), 6.21 (1 H, d, C-5, J = 2.5 Hz), 6.56 (1 H, d, C-3, J = 2.5 Hz), and 7.17 (1 H, d, C-1′, J = 15 Hz). ^{13}C NMR ($CDCl_3$): δ 21.30 (C-11′), 21.70 (C-8′), 22.80 (C-4′), 30.60 (C-3′), 32.10 (C-9′), 35.10 (C-7′), 36.70 (C-5′), 66.90 (C-6′), 73.80 (C-10′), 102.50 (C-3), 103.80 (C-1), 108.80 (C-5), 132.90 (C-2′), 133.60 (C-1′), 144.50 (C-6), 160.47 (C-4), 165.70 (C-2), and 171.70 (C-12′).

β-Zearalenol (3) was produced by *Mucor bainieri* NRRL 2988 in 60% yield as well as by other microorganisms in lower yield (Table I): mp (from $CHCl_3$–hexane) 173–174 °C [lit.[17] mp 174–176 °C], R_f 0.54, t_R 12.2 min. IR (KBr): 3500, 3180, 1630, 1610, and 968 cm^{-1}. Mass spectrum (direct probe, 23 eV): m/e 320 (M^+), 302, 233, 225, 188 (100%), 112, and 97. The TMS derivative spectrum displayed a molecular ion m/e 536 (M^+), 464, 446 (100%), 320, 305, 260, 196, and 169. ^{1}H NMR (acetone-d_6): δ 1.46 (3 H, d, C-11′), 1.62 (2 H, m, C-8′), 1.90 (2 H, m, C-4′), 2.25 (2 H, m, C-3′), 2.30 (2 H, m, C-9′), 2.85 (2 H, m, C-7′), 3.48 (1 H, m, C-5′), 3.76 (2 H, m, C-6′), 5.10 (1 H, m, C-10′), 5.98 (1 H, m, C-2′), 6.34 (1 H, d, C-5, J = 2.5 Hz), 6.54 (1 H, d, C-3, J = 2.5 Hz), and 6.90 (1 H, d, C-1′, J = 15 Hz). ^{13}C NMR ($CDCl_3$): δ 18.98 (C-11′), 18.73 (C-8′), 21.58 (C-4′), 30.34 (C-3′), 30.84 (C-9′), 33.81 (C-7′), 35.32 (C-5′), 66.95 (C-6′), 72.58 (C-10′), 102.60 (C-3), 103.82 (C-1), 108.57 (C-5), 131.71 (C-2′), 133.58 (C-1′), 144.58 (C-6), 160.80 (C-4), 164.97 (C-2), and 170.30 (C-12′).

α-Zearalanol (4) was produced in 20% yield by *Aspergillus ochraceous* NRRL 405: mp (from $CHCl_3$–hexane) 181–182 °C [lit.[16] mp 182–183 °C], R_f in normal phase 0.49 and in reverse phase 0.51. IR (Nujol): 3500, 3180, 1645, and 1620 cm^{-1}. Mass spectrum (direct inlet): m/e 322 (M^+), 304, 286, 236, 207, 206, 169 (100%), 164, 163, 150, and 99. The TMS derivative showed m/e 538 (M^+), 467, 454, 434 (100%), 395, 379, 352, 336, 323, 321, 307, 268, 171, 129, and 81. ^{1}H NMR spectrum (acetone-d_6): δ 1.34 (3 H, d, C-11′), 1.52 (2 H, m, C-8′), 1.66 (2 H, m, C-4′), 1.86 (2 H, m, C-3′), 2.10 (2 H, m, C-2′), 2.42 (2 H, m, C-9′), 3.21 (2 H, m, C-7′), 3.25 (2 H, m, C-5′), 3.58 (2 H, d, C-1′), 3.76 (1 H, t, C-6′), 5.17 (1 H, m, C-10′), 6.22 (1 H, d, C-5), and 6.28 (1 H, d, C-3). ^{13}C NMR: δ 20.90 (C-11′), 24.01 (C-8′), 27.05 (C-4′), 32.71 (C-2′), 33.09 (C-3′), 34.31 (C-9′), 34.66 (C-7′), 35.90 (C-5′), 38.15 (C-1′), 66.85 (C-6′), 73.70 (C-10′), 102.34 (C-3), 103.26 (C-1), 109.21 (C-5), 145.15 (C-6), 161.02 (C-4), 165.98 (C-2), and 171.20 (C-12′).

β-Zearalanol (5). A 50% transformation was obtained by *Aspergillus niger* X-170: mp (from $CHCl_3$–hexane) 152–153 °C [lit.[16] mp 145–147 °C]; R_f 0.51 and 0.52 in normal and reverse phase TLC, respectively. IR spectrum (Nujol): 3500, 1680, 1650, and 1622 cm^{-1}. ^{1}H NMR (CD_3OD): δ 1.33 (3 H, d, C-11′), 1.50 (2 H, m, C-8′), 169 (2 H, m, C-4′), 1.85 (2 H, m, C-3′), 2.15 (2 H, m, C-2′), 2.65 (2 H, m, C-9′), 3.19 (2 H, m, C-7′), 3.21 (2 H, m, C-5′), 3.50 (2 H, d, C-1′), 3.77 (1 H, m, C-6′), 5.18 (1 H, m, C-10′), 6.29 (1 H, d, C-5), and 6.36 (1 H, d, C-3). ^{13}C NMR (CD_3OD): δ 21.40 (C-11′), 23.91 (C-8′), 26.80 (C-4′), 31.96 (C-2′), 32.25 (C-9′), 32.85 (C-3′), 33.20 (C-7′), 35.10 (C-5′), 37.90 (C-1′), 67.21 (C-6′), 73.68 (C-10′), 102.21 (C-3), 103.60 (C-1), 108.56 (C-5), 145.42 (C-6), 160.90 (C-4), 165.80 (C-2), and 170.90 (C-12′).

Zearalanone (6) was produced in 20% yield by *Saccharomyces cerevisiae* NRRL Y2034: mp (from $CHCl_3$–hexane) 190–191 °C [lit.[16] mp 192–193 °C]; R_f 0.79 in normal phase TLC. IR spectrum (KBr): 3500, 3180, 1750, 1650, and 1620 cm^{-1}. Mass spectrum (direct inlet) showed a molecular peak: m/e 320 (M^+), 302, 169, 163, 125, and 112 (100%). ^{1}H NMR (CD_3OD): δ 1.33 (3 H, d, C-11′), 1.57 (2 H, m, C-8′), 1.76 (2 H, m, C-4′), 1.89 (2 H, m, C-3′), 2.11 (2 H, m, C-9′), 2.32 (2 H, m, C-2′), 2.69 (2 H, d, C-7′), 3.00 (2 H, t, C-1′), 3.10 (2 H, d, C-5′), 5.18 (1 H, m, C-10′), 6.16 (1 H, d, C-5), and 6.18 (1 H, d, C-3). ^{13}C NMR (CD_3OD): δ 21.71 (C-11′), 23.88 (C-8′), 28.93 (C-4′), 32.08 (C-2′), 33.04 (C-3′), 36.49 (C-9′), 37.83 (C-1′), 39.81 (C-7′), 45.09 (C-5′), 73.98 (C-10′), 102.33 (C-3), 103.18 (C-1), 112.41 (C-5), 149.66 (C-6), 164.16 (C-4), 166.97 (C-2), 173.10 (C-12′), and 215.37 (C-6′).

8′(*S*)-Hydroxyzearalenone (7) was produced in 32% yield using *Streptomyces rimosus* NRRL 2234: mp (from acetone–hexane) 173–174 °C [lit.[20] mp 172–174 °C]; $[\alpha]_D$ −53.2 (c 1, acetone); R_f 0.55. IR (KBr): 3530, 3300, 3180, 1690, 1640, 1612, and 980 cm^{-1}. Mass spectrum (direct probe): m/e 334 (M^+), 316, 248, 204, 188, 161 (100%), 110, and 95. TMS derivative gave rise to m/e 550 (M^+), 478, 460, 442, 391, 377, 333 (100%), and 305. ^{1}H NMR ($CDCl_3$): δ 1.04 (3 H, d, C-11′), 1.34 (2 H, m, C-4′), 1.48 (1 H, m, C-3′), 1.99 (2 H, m, C-9′), 2.35 (2 H, m, C-7′), 2.77 (2 H, m, C-5′), 3.71 (1 H, m, C-8′), 4.25 (1 H, m, C-10′), 6.30 (1 H, d, C-2′), 6.82 (1 H, d, C-5, J = 2.51 Hz), 7.06 (1 H, d, C-3, J = 2.51 Hz), and 7.19 (1 H, d, C-1′). ^{13}C NMR (DMSO-d_6): δ 21.00 (C-11′), 23.10 (C-4′), 31.20 (C-3′), 39.10 (C-9′), 43.30 (C-5′), 51.70 (C-7′), 64.60 (C-8′), 69.80 (C-10′), 102.05 (C-3), 102.80 (C-1), 108.40 (C-5), 131.00 (C-2′), 144.20 (C-6), 164.00 (C-4), 166.11 (C-2), 172.10 (C-12′), and 212.10 (C-6′); mass spectrum, calcd for $C_{18}H_{22}O_6$ m/e 334.3682, found m/e 334.3698.

Zearalenone 2,4-bis(methyl ether) (8) was obtained by using *Cunninghamella bainieri* ATCC 9244B in 25% yield: mp (from acetone–hexane) 111–112 °C [lit.[16] mp 112–114 °C]; R_f 0.59. IR (Nujol) showed absorption maxima at 1725, 1600, 1455, and 960 cm^{-1}. Mass spectrum (direct inlet): m/e 346 (M^+), 328, 235, 217 (100%), 207, 204, 189, 151, 125, and 112. ^{1}H NMR ($CDCl_3$): δ 1.34 (3 H, d, C-11′), 1.71 (2 H, m, C-8′), 1.75 (2 H, m, C-4′), 2.12 (2 H, m, C-3′), 2.27 (2 H, m, C-9′), 2.40 (2 H, m, C-7′), 2.70 (2 H, t, C-5′), 3.79 (3 H, s, C_4-OCH_3), 3.81 (2 H, s, C_2-OCH_3), 5.31 (1 H, m, C-10′), 5.99 (1 H, m, C-2′), 6.37 (1 H, d, C-5), 6.39 (1 H, d, C-1′), and 6.59 (1 H, d, C-3). ^{13}C NMR ($CDCl_3$): δ 20.08 (C-11′), 21.35 (C-8′), 21.45 (C-4′), 31.29 (C-3′), 35.21 (C-9′), 37.65 (C-7′), 44.09 (C-5′), 55.44 (C_4-OCH_3), 55.47 (C_2-OCH_3), 71.20 (C-10′), 97.79 (C-3), 101.39 (C-5), 116.31 (C-1), 129.04 (C-1′), 133.21 (C-2′), 136.84 (C-6), 157.74 (C-2), 160.85 (C-4), 167.59 (C-12′), and 211.35 (C-6′); mass spectrum, calcd for $C_{20}H_{26}O_5$ m/e 346.4224, found m/e 346.4228.

Zearalenone 2-methyl ether (9) was produced by conversion of zearalenone using *Cunninghamella bainieri* ATCC 9244B in 22% yield: mp (from acetone–hexane) 189–191 °C [lit.[16] mp 190–192 °C]; R_f 0.51. IR spectrum (KBr) showed absorption maxima: 1725, 1605, 1599, 1580, and 965 cm^{-1}. Mass spectrum (direct inlet): m/e 332 (M^+), 314, 217 (100%), 193, 175, 151, 125, and 112. ^{1}H NMR ($CDCl_3$): δ 1.34 (3 H, d, C-11′), 1.61 (2 H, m, C-8′), 1.79 (2 H, m, C-4′), 2.18 (2 H, m, C-3′), 2.22 (2 H, m, C-9′), 2.42 (2 H, m, C-7′), 2.73 (2 H, t, C-5′), 3.81 (3 H, s, C_2-OCH_3), 5.29 (1 H, m, C-10′), 5.91 (1 H, m, C-2′), 6.31 (1 H, d, C-5), 6.34 (1 H, d, C-1′), and 6.51 (1 H, d, C-3). ^{13}C NMR ($CDCl_3$): δ 20.05 (C-11′), 21.29 (C-8′), 21.83 (C-4′), 31.32 (C-3′), 35.15 (C-9′), 37.73 (C-7′), 44.08 (C-5′), 55.05 (C_2-OCH_3), 71.44 (C-10′), 98.27 (C-3), 104.02 (C-5), 116.63 (C-1), 128.76 (C-1′), 133.25 (C-2′), 136.88 (C-6), 157.94 (C-2), 162.80 (C-4), 168.47 (C-12′), and 211.38 (C-6′); mass spectrum, calcd for $C_{19}H_{24}O_5$ m/e 332.3956, found m/e 332.3914.

Estrogen Receptor Binding Assay. Fresh rat uteri, stored in ice-cold saline, were freed of adherent fat and connective tissue at 4 °C. After addition of TEMG buffer (3–4 uteri/mL), the uteri were homogenized by treatment with a Teflon-glass homogenizer until 90% of the uteri were disrupted. The homogenate was centrifuged at 105 000 × g for 1 h (0 °C). The supernatant was then diluted with TED buffer (1:2) to obtain the cytosolic fraction that was used for determining the affinity of the compounds for the estrogen receptor. The protein concentration of the cytosol was about 12 mg/mL, leading to a final concentration of 6 mg/mL in the assay.

For the determination of the RBA, the previously described procedure was applied with modification.[25] Aliquots of uterine cytosol (150 μL) were added to a mixture of 50 μL of 4 nM

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^{3}H-estradiol [2,4,6,7-^{3}H(estradiol), 110 Ci/mmol), New England Nuclear] in TED buffer and 50 μL of 10^{-10}–10^{-5} M of competing ligand. The mixture was incubated for 18 h at 4 °C, and then 0.3 mL of DCC slurry (0.8% charcoal Norit A and 0.008% dextran in TED) was added to the tubes, and the contents were mixed. The tubes were incubated for 15 min at 4 °C and centrifuged at 800 × g for 10 min. An aliquot (200 μL) of the supernatant was removed and the radioactivity quantified by liquid scintillation spectrometry after addition of 5 mL of Aquasol-2 (New England Nuclear). Nonspecific binding was calculated by using 5 μM 17β-estradiol as competing ligand. Radioactivity was plotted as a function of the log concentration of competing ligand in the assay. The RBA was calculated as the ratio of the molar concentrations of estradiol and test compound required to decrease the amount of bound radioactivity by 50%, multiplied by 100.

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Electrophilic Substitution Reaction at Azomethine Carbon Atom. Acylation of Aliphatic Aldehyde Hydrazones

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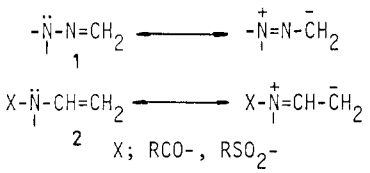
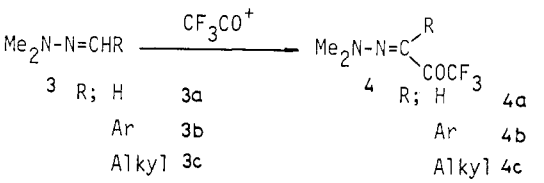
Formaldehyde dialkylhydrazones were found to undergo an electrophilic substitution reaction very easily at the azomethine carbon atom when treated with trifluoroacetic anhydride. Bistrifluoroacetylation is also possible. As an interesting extension of this reaction, trifluoroacetylation of dialkylhydrazones of aliphatic aldehydes and α,β-unsaturated aldehydes, was achieved. Similar reactions of these hydrazones with some other acylating reagents were also studied.

Introduction

Recent studies in this laboratory on the successful electrophilic substitution reaction at olefinic carbon atoms[1-8] prompted us to extend these works to hydrazone system. As for C=N linkage, hydrazones are quite different from imines in electronic character. In the case of imines, the C=N bond is polarized with positive charge at carbon and negative charge at nitrogen according to their inherent electronegativities. In contrast, in the case of hydrazone 1, conjugation of the C=N bond with adja-



cent nitrogen makes the azomethine carbon negatively charged. In this sense the azomethine carbon of hydrazone **1** is akin to the terminal olefinic carbon atoms of *N*-vinylcarboxamides or *N*-vinylsulfonamides **2**[3] and is expected to be acylated easily. In fact it was reported in our preceding paper[9] that the dimethylhydrazone of aromatic aldehydes **3b** can react with trifluoroacetic anhydride



(TFAA) at the azomethine carbon, i.e., electrophilic substitution reaction occurs there successfully. In order to establish this type of reaction more definitely, it become necessary to examine it with hydrazones of aliphatic aldehydes. As the simplest case, trifluoroacetylation of dimethylhydrazone of formaldehyde was studied first. Unusual resistance of **3c** toward TFAA was experienced in our preliminary experiments[9] and was the important problem to be solved in the present work. Reaction of **3** toward some other acylating reagents also attracted our interest.

Conversion of **3** to **4** is a key step of a series of transformations[9] from aldehydes to 1,2-dicarbonyl compounds **5**, as is illustrated in Scheme I.

Results and Discussion

Trifluoroacetylation of Formaldehyde Dimethylhydrazone (3a). On the basis of our semiempirical MO calculation carried out previously,[10] the reactivity of the azomethine carbon of **6a** toward electrophiles was estimated to be comparable to that of the β-carbon of **6b** as

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(10) Unpublished results.

(11) Our calculations carried out on the basis of CNDO/2 and MINDO/3 methods suggest the following. Although frontier electron density (HOMO) at the azomethine carbon of **6a** is estimated to be slightly less than that at the olefinic β-carbon of **6b**, the HOMO level of **6a** is higher than that of **6b**.

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