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Enantioseparation of Vinclozolin by γ-Cyclodextrin-Modified Micellar Electrokinetic Chromatography

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Cyclodextrin-modified micellar electrokinetic chromatography was applied to the enantioseparation of vinclozolin, which has been used as a fungicide and has an anti-androgenic activity, using γ-cyclodextrin together with sodium dodecyl sulfate. Factors affecting the chiral resolution and migration time of vinclozolin were studied. The optimum running conditions were found to be 20 mM phosphate−5 mM borate buffer (pH 8.5) containing 50 mM γ-cyclodextrin and 100 mM sodium dodecyl sulfate with an effective voltage of 20 kV at 20 °C using direct detection at 203 nm. Under these conditions, the resolution (Rs) of racemic vinclozolin was approximately 2.1. The sample was concentrated by solid-phase extraction and was fractionated by HPLC. The peak area ratio of (+)- and (−)-vinclozolins in wine was found to be 2:3, namely, not racemic, suggesting that degradation rates were different between (+)- and (−)-vinclozolins. The anti-androgenic activities of (+)- and (−)-vinclozolins on dihydrotestosterone-induced transcription were also investigated. The anti-androgenic activity of (+)-vinclozolin tended to be stronger than that of (−)-vinclozolin, suggesting the possibility that vinclozolin can act as an enantioselective anti-androgen.

KEYWORDS: Enantioseparation; micellar electrokinetic chromatography; vinclozolin; wine; anti-androgenic activity

INTRODUCTION

Capillary electrophoresis (CE) is a recently developed powerful analytical technique with a wide range of applications. As demonstrated by Terabe (*1*, *2*), the introduction of micelles, which serve as a pseudophase, into the separation electrolyte results in the separation of neutral species. Several methods have been tried for enantioseparation of neutral analytes by CE. One method is to add a chiral surfactant solution or a mixed micelle solution of chiral and achiral surfactants to the buffer solution. The first chiral separation by micellar electrokinetic chromatography (MEKC) was reported by Cohen et al. (*3*), who used *N*,*N*-didecyl-L-alanine as a chelating surfactant together with sodium dodecyl sulfate (SDS) and Cu^{2+} to form mixed micelles having a chiral ligand. The difference in the formation constants led to the enantioseparation of dansylated amino acids. Subsequently, chiral surfactants have been successfully employed for chiral separations by MEKC using optically active amino acid-derived synthetic surfactants (*4*, *5*) or natural surfactants such as bile salts (*6*) and digitonin (*7*). Another method is to use a buffer containing a single isomer of charged cyclodextrin or a mixture of neutral and charged cyclodextrins as previously reviewed (*8*−*10*).

Chiral compounds have also been separated by MEKC by adding cyclodextrin (CD) to the buffer solution as recently reviewed (*9*, *11*−*14*). This method is called CD-modified MEKC (CD-MEKC). In CD-MEKC, a neutral solute is partitioned between the micellar and the aqueous CD phases. When the solute is included in the CD cavity or is dissolved in the electrolyte, it migrates with the electroosmotic velocity. And when it is incorporated into the micelles, it migrates with the micellar velocity. The solutes can be separated by their differential partition between the micellar and the aqueous CD phases. Accordingly, an analyte having a shorter migration time is included more strongly in the CD cavity than an analyte having a longer migration time.

Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione] (**Figure 1**) is a dicarboximide fungicide that is effective in the control of diseases caused by *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Moniliniam* spp. It has been widely used in Europe to protect fruits, vegetables, ornamental plants, and turf grasses. A number of investigations on the determination of multiresidues containing vinclozolin have been conducted by gas chromatography using nitrogen−phosphorus detection, electron-capture detection, or mass spectrometry (*15*−

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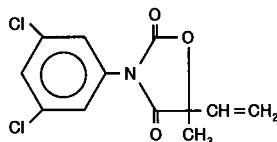

Figure 1. Structure of vinclozolin.

22). HPLC techniques have also been proposed (*23*−*27*). Using the above methods, vinclozolin has been detected in foods and soil. Recently, we have reported the rapid determination of six pesticides including vinclozolin in wine by gas chromatography using an electron-capture detector with a large-volume injection system (*28*). Vinclozolin, having an asymmetric carbon, has been used as a racemic compound. However, to our knowledge, there is no report on the enantiomeric separation and enantioselective biological activity of vinclozolin.

The aim of the present study was to develop a chiral separation of vinclozolin using CD-MEKC and to determine the ratio of (+)- and (−)-vinclozolin residues in wine. We have also examined the anti-androgenic activity of (+)- and (−)-vinclozolins.

MATERIALS AND METHODS

Chemicals. Sodium dodecyl sulfate (electrophoresis purity reagent) was obtained from Bio-Rad. 2-Hydroxypropyl-β-cyclodextrin (average degree of substitution 7) was obtained from Sigma. Hydroxypropyl-γ-cyclodextrin (average degree of substitution 4.2) was obtained from Aldrich. γ-Cyclodextrin, vinclozolin, and other chemicals (analytical grade) were obtained from Wako Pure Chemical Industries Ltd.

CE Apparatus. Electrophoretic experiments were carried out using a capillary electrophoresis system (G1600A, Agilent Technologies). Samples were injected by a pressure of 50 mbar for 2 s. The separations were performed in a fused silica bubble cell capillary of 64.5 cm (effective length 56 cm) × 75 μm Ø (Agilent Technologies). The capillary was kept at 20 °C. The analytes were detected at 203 nm. The power supply was operated in the constant-voltage mode, at 20 kV, and the substances migrated toward the negative pole.

HPLC Apparatus. The HPLC system consisted of a Hitachi model L-6300 pump, a Rheodyne manual injector, a Shimadzu photodiode array detector (model SPD-M10AV), and a Shimadzu column oven (model CTO-10AC). Achiral separation was attained using an RSpak DE-613 column (6 mm Ø × 150 mm, Showa Denko). For the chiral resolution, a Chiralcel OC column (4.6 mm Ø × 250 mm, Daicel Chemical Industries, Ltd.) and both a Shimadzu photodiode array detector and a Jasco model OR-990 polarimetric detector were used. Data acquisition and processing were conducted with a Shimadzu LC model CLASS-M10A workstation.

Preparation of Vinclozolin Enantiomers. Vinclozolin enantiomers were separated by HPLC using a Chiralcel OC column thermostated at 30 °C. Hexane−ethanol (98:2) was used as the mobile phase, and the flow rate was 1 mL/min. Two fractions, corresponding to the (+)- and (−)-enantiomers of vinclozolins, were separately collected. When the enantiomeric excess (ee) is defined as the difference between the amount of the two enantiomers in a mixture divided by their total, the purities of (+)- and (−)-vinclozolins collected are more than 99.8% ee and 99.0% ee, respectively.

Buffer and Sample Preparation for CE. The background electrolyte (BGE) in the electrophoretic experiments, unless stated otherwise, was 20 mM phosphate−5 mM borate buffer (pH 8.5) containing 100 mM SDS and 50 mM γ-CD and was filtered with a 0.22 μm filter before use. Purified water was obtained from a Toray ultrapure water system.

Stock solutions of 1000 mg/L racemic (+)- and (−)-vinclozolins were separately prepared in acetonitrile, stored at −20 °C, and diluted to 30 mg/L before use.

Incubation of Vinclozolin in Aqueous Buffers. A 1 mL sample of racemic vinclozolin at 50 mg/L in acetonitrile was mixed with 9 mL of 11 mM acetate buffer (pH 4.0) or 11 mM Tris−HCl buffer (pH 7.4) and was incubated at 35 °C. After various intervals of incubation, the concentrations of (+)- and (−)-vinclozolins were analyzed by the above CE method.

Calculation of Resolution. The resolution (Rs) of the enantiomer was calculated by using the equation

$$Rs = 2(t_2 - t_1)/(w_1 + w_2)$$

where t is the migration time and w is the width of the peak at the baseline.

Enantiomeric Analysis of Vinclozolin in Wine. Wine (20 mL) was applied to a Sep-Pak Plus PS-2 cartridge, and this process was repeated 24 more times. The cartridges were washed with water and were dried under vacuum by aspiration. The absorbed materials were eluted with acetonitrile. All the eluents were combined and were concentrated to dryness under rotary vacuum evaporation. The resulting residue was dissolved in 0.3 mL of acetonitrile. The extractant (each 50 μL, six times) was applied to an RSpak DE-613 column maintained at 40 °C. The mobile phase used was 62% (v/v) acetonitrile, and the flow rate was 1.5 mL/min. The fraction containing vinclozolin was combined and diluted with water. The diluted solution was applied to a Sep-Pak Plus PS-2 cartridge. The cartridge was washed with water, and the absorbed materials were eluted with acetonitrile. The eluent was concentrated to dryness under rotary vacuum evaporation. The resulting residue was dissolved in 0.1 mL of 20% (v/v) acetonitrile and was analyzed by the above CE method.

Cell Culture. The PC3/AR cell line derived from human prostate carcinoma PC-3 stably expresses human wild-type androgen receptor (*29*). PC3/AR cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2−95% air. In routine maintenance, PC3/AR cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 mg/L streptomycin, and 5000 units/L penicillin and passaged with trypsinization every fourth day.

Luciferase Assay. PC-3/AR cells were plated on 24-well culture plates at a density of 5 × 10^4 cells/well and cultured for 10 h. The medium was replaced with serum-free and antibiotic-free OPTI-MEM medium (Life Technologies), and the cells were transfected using a cationic lipid-mediated system with 0.5 μL of LIPOFECTIN reagent (Life Technologies) and either 200 ng of an androgen receptor-driven luciferase expression vector plasmid pGLPSAp5.8 (*30*) or 200 ng of empty expression vector pGL3-Basic (Promega) for 20 h. The medium was replaced with phenol-red-free RPMI-1640 medium supplemented with 5% charcoal/dextran-treated fetal bovine serum (Hyclone), and the cells were treated with vehicle (ethanol) or a test compound in the presence of 0.5 nM 5α-dihydrotestosterone (DHT) for 36 h. Then, the cells were rinsed twice with cold phosphate-buffered saline and lysed with 80 μL of PicaGene cell lysis reagent Luc (Toyo Ink). Cell lysates were collected by scraping and placed into microcentrifuge tubes and centrifuged at 12000 rpm for 15 min at 4 °C. A 20 μL sample of supernatant was combined with 100 μL of PicaGene luminescence reagent (Toyo Ink), and luminescence was measured immediately using a Berthold luminometer (model Lumat LB9501). Luciferase assays were performed in quintuplicate, and relative light units were normalized to protein content. The protein content was measured by the Bradford method (*31*).

Statistical Analysis. Statistical analyses were made using an unpaired Student's *t*-test with StatView-J 5.0 for a Macintosh computer (Nankodo), whereby a value of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Factors Affecting Chiral Separation. The effect of the type of CD on the enantioseparation of vinclozolin was investigated by CD-MEKC using a BGE containing separately 50 mM 2,6-di-*o*-methyl-β-CD, 2,3,6-tri-*o*-methyl-β-CD, 2-hydroxypropyl-β-CD, hydroxypropyl-γ-CD, or γ-CD. In the absence of CD, there was no enantioseparation and the analytes were detected as a single peak (**Figure 2A**). The addition of hydroxypropyl-γ-CD in a BGE did not affect the enantioseparation (**Figure

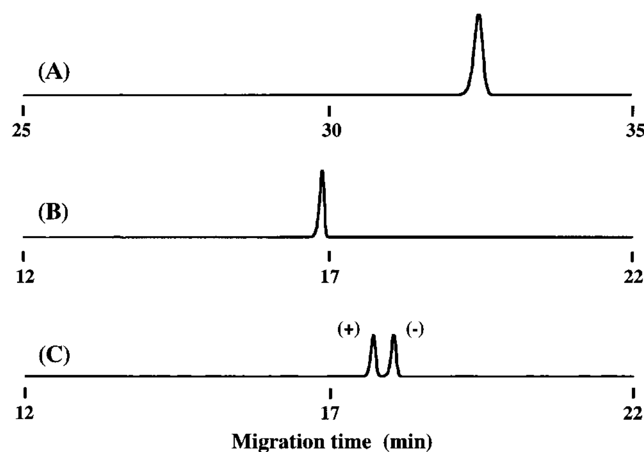

Figure 2. Electropherograms of racemic vinclozolin obtained by MEKC using CDs. The BGE was composed of 100 mM SDS and 20 mM phosphate–5 mM borate buffer (pH 8.5) with no CD (A), 50 mM hydroxypropyl-γ-CD (B), or 50 mM γ-CD (C). (+) and (–) represent (+)- and (–)-vinclozolin, respectively.

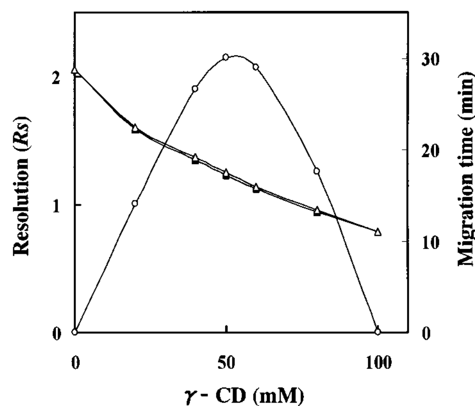

Figure 3. Effect of γ-CD concentration on the resolution and migration time of vinclozolin: (○) resolution (Rs); (△) migration time of (+)-vinclozolin. The BGE was composed of various concentrations of γ-CD containing 100 mM SDS and 20 mM phosphate–5 mM borate buffer (pH 8.5).

2B) and resulted in a shorter migration time than that in the absence of CD, suggesting that both vinclozolin enantiomers have the same partition between hydroxypropyl-γ-CD and the SDS micelles. Similar results were obtained with β-CD derivatives. On the other hand, γ-CD had a dramatic effect on the separation of vinclozolin enantiomers (**Figure 2C**). According to Terabe et al. (*32*), in CD-MEKC, an analyte is distributed among three phases, the micellar phase, the CD phase, and the aqueous phase excluding the micelles and CD, although CD is not a true phase. As γ-CD is electrically neutral, γ-CD migrates at the same velocity as the bulk solution. The distribution of the analyte between the micelles and the nonmicellar aqueous phase including γ-CD directly affects the resolution. γ-CD added to the micellar solution reduces the partitioning of the analyte to the micelles by increasing the fraction of the analyte in the nonmicellar aqueous phase. This indicates that (+)-vinclozolin formed a more stable complex with γ-CD than did (−)-vinclozolin.

The effect of the γ-CD concentration (0−100 mM) on the resolution and migration time of vinclozolin was studied (**Figure 3**). The migration time decreased with increasing amounts of γ-CD, suggesting that a higher γ-CD concentration formed a more stable complex of vinclozolin with γ-CD. The resolution of vinclozolin showed a maximum at 50 mM. Wren and Rowe (*33*) developed a theoretical model relating mobility to the concentration of a CD selector. They suggested that the maximum resolution can be obtained at the optimum CD concentration, $C_{opt} = (K_A K_B)^{-1/2}$, where K_A and K_B are formation constants for inclusion complexes of CD with enantiomers A and B, respectively. The optimum CD concentration was also found in the γ-CD-MEKC.

The effect of SDS concentration (60−150 mM) on the resolution and migration time of vinclozolin was studied. The resolution increased with increasing SDS concentration of the BGE up to 50 mM and then decreased gradually. An increase in the concentration of SDS brought about an increase in the migration time. This result is not attributable to an increase in ionic strength, because the electroosmotic flow was not changed significantly over the whole SDS concentration range. The partition of the solute between the CD and the micelles depends on the SDS concentration. This suggests that the longer migration time resulted from an increase in the phase ratio of the micellar to the aqueous CD phase.

The effect of the pH (7.5−9.5) of the BGE on the resolution and migration time of vinclozolin was studied. The resolution was not affected by the pH of the BGE at all. The migration time slightly decreased with an increasing pH. It is well-known that vinclozolin is unstable in aqueous solution. In fact, when racemic vinclozolin was incubated in aqueous solution at pH 4.0 and 7.5, peaks of (+)- and (−)-vinclozolins decreased with an increase in incubation time, and two doublet peaks that resulted from degradation of vinclozolin were detected at migration times of 11 and 12 min. However, in the enantioseparation of a standard vinclozolin solution by the proposed method, these doublet peaks were not detected.

The effect of capillary temperature (15−35 °C) on the resolution and migration time of vinclozolin was studied. A lower capillary temperature caused increases in both the resolution and migration time of vinclozolin. According to Heuermann and Braschke (*34*), the increase in the Rs value with a decrease in temperature might be explained by a decrease in the rotational and/or vibrational energy, increasing the fixation of the enantiomers to the inside or to the rim of CD and, thus, increasing the enantioselectivity. The shorter migration time at higher temperature could be attributed to a decrease of the buffer viscosity (*35*).

Therefore, the optimum BGE conditions, i.e., the conditions giving both high resolution and short migration time, were found to be 50 mM γ-CD and 100 mM SDS in 20 mM phosphate−5 mM borate buffer (pH 8.5) with an effective voltage of 20 kV at 20 °C.

Enantioseparation of Vinclozolin in Wine. Racemic vinclozolin (0.3−30 mg/L) was subjected to the CE method using the above optimum conditions. Linearity ($r^2 > 0.999$) was demonstrated in the range 0.5−15 mg/L by standard curves of each (+)- and (−)-vinclozolin. The reproducibility of five consecutive determinations was evaluated at 3 mg/L for racemic vinclozolin. High reproducibilities of the peak areas (RSD < 1.3%) and migration times (RSD < 1.0%) of both (+)- and (−)-vinclozolins were obtained.

Using the proposed CE method, (+)- and (−)-vinclozolins in two brands of wines were analyzed. Since vinclozolin is a medium polar compound, we tried to examine solid-phase extraction using a PS-2 cartridge, which is packed with a poly(methacrylate)-based material and which strongly adsorbs medium to highly polar compounds, to concentrate vinclozolin in wine. Wine contains approximately 15% ethyl alcohol and many other ingredients. In our preliminary experiment, the

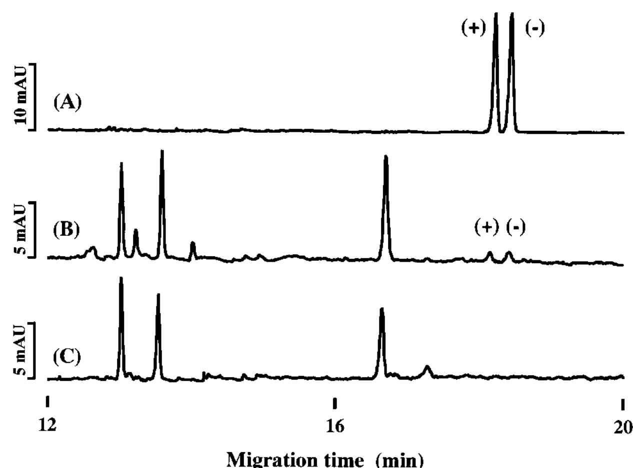

Figure 4. Electropherograms of wines: (A) racemic vinclozolin; (B, C) red wines.

amount of wine loaded onto the cartridge, on which vinclozolin can be quantitatively adsorbed, was maximally 20 mL. Since it seemed that vinclozolin was present in wine at sub-ppb levels, we repeated the solid extraction 25 times. First, the eluent was applied to the CE method. However, there were too many peaks to analyze vinclozolin enantiomers. Thus, the eluent was applied to an RSpak DE-613 column, and the fraction containing vinclozolin was collected. **Figure 4** shows the electropherograms of the fractions obtained from the two wines. Vinclozolin was detected at the sub-ppb level in one of the two wines. The peak area ratio of (+)- and (−)-vinclozolins was 2:3, respectively; thus, vinclozolin residue in wine was found to be not racemic. Szeto et al. (*23*) studied the hydrolysis of vinclozolin in aqueous buffers of pH 4.5−8.3 at 35 °C. All data for the hydrolysis of vinclozolin followed simple pseudo-first-order kinetics. The reaction was base-catalyzed, and the rate was found to be proportional to the pH. We also examined the hydrolysis of racemic vinclozolin in aqueous buffers of pH 4.0 or 7.4 at 35 °C. As with the above results, the disappearance of vinclozolin was very fast at pH 7.4 but much slower at pH 4.0. It was also found that (+)-vinclozolin disappeared at the same rate as the (−)-isomer at both pH 4.0 and pH 7.4. The present CE study suggests that (+)-vinclozolin is degraded faster than (−)-vinclozolin by biological actions during the wine-making process.

Anti-Androgenic Activities of (+)- and (−)-Vinclozolin. The effect of (+)- and (−)-vinclozolins on DHT (a natural androgen)-induced transcription was investigated in PC3/AR cells transiently transfected with AR-driven luciferase-expressing vector plasmid pGLPSAp5.8 which was constructed by Mizokami et al. (*30*). Transcriptional activity induced with 10 pM DHT decreased with increasing amounts of racemic (+)- or (−)-vinclozolin (**Table 1**). The anti-androgenic activity of (+)-vinclozolin tended to be stronger than that of (−)-vinclozolin, while significant variation was not observed between the activities of (+)- and (−)-vinclozolins. Clark (*36*) reported that vinclozolin was unstable in methanolic and ethanolic solutions and water suspensions and that vinclozolin metabolites did not inhibit mycelial growth of *Botrytis cinerea* in vitro, indicating that they have no antifungal activity. Three hydrolytic degradation products of vinclozolin were identified, namely, 2-[[(3,5-dichlorophenyl)carbamoyl]oxy]-2-methyl-3-butenoic acid (M1), 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), and 3,5-dichloroaniline (M3), and a degradation pathway of vinclozolin in aqueous solution was proposed (*23*−*25*). Administration of vinclozolin to pregnant rats caused incomplete development of the male reproductive tract in male pups (*37*), indicating anti-androgenic activity. Vinclozolin was a poor inhibitor of androgen binding to rat androgen receptor in cell-free extracts, whereas M1 and M2 were effective competitors (*38*), suggesting that the anti-androgenic effects of vinclozolin were mediated by M1 and/or M2. Subsequently, Wong (*39*) reported that M1 and M2, in a dose-dependent manner, targeted androgen receptor to the nucleus and inhibited androgen-induced transactivation mediated by the mouse mammary tumor virus promoter and that M2 was a 50-fold more potent inhibitor than M1. Both M1 and M2 as well as vinclozolin have an asymmetric carbon. As described above, peaks of both (+)- and (−)-vinclozolins decreased at the same rate with incubation time. This result could mean that, during incubation in aqueous solution, the amounts of M1 and M2 enantiomers resulting from (+)-vinclozolin were the same as those resulting from (−)-vinclozolin. Recently, the binding affinities of chiral ligands to receptors have been studied, indicating that one enantiomer is significantly more active than the other (*40*−*43*). This suggests that M1 and M2 enantiomers produced from (+)-vinclozolin bound more strongly to the androgen receptor than did those produced from (−)-vinclozolin.

Table 1. Anti-Androgenic Activities of Racemic (+)- and (–)-Vinclozolins

compd		activity (×1000) (relative light unit)
blank		23 ± 6
positive control (10 pM DHT)		393 ± 126
10 pM DHT		
+ racemic vinclozolin	0.05 μM	396 ± 62
	0.5 μM	226 ± 49
	5 μM	72 ± 30
+ (+)-vinclozolin	0.05 μM	348 ± 133
	0.5 μM	196 ± 61
	5 μM	63 ± 16
+ (–)-vinclozolin	0.05 μM	339 ± 52
	0.5 μM	287 ± 91
	5 μM	78 ± 23

CONCLUSION

Direct enantioseparation of vinclozolin was performed by γ-CD-MEKC. The enantioseparation was based upon solid-phase extraction and fractionation with HPLC followed by the MEKC. The peak area ratio of (+)- and (−)-vinclozolins in wine was found to be 2:3, namely, not racemic, suggesting that the degradation rates during the wine-making process were different between (+)- and (−)-vinclozolins. The anti-androgenic activity of (+)-vinclozolin tended to be stronger than that of (−)-vinclozolin. This suggests that M1 and M2 enantiomers produced from (+)-vinclozolin bound more strongly to the androgen receptor than did those produced from (−)-vinclozolin.

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