Mouse Steroid 15α-Hydroxylase Gene Family: Identification of Type II P-450$_{15\alpha}$ as Coumarin 7-Hydroxylase

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ABSTRACT: We identified type II P-450$_{15\alpha}$ as mouse coumarin 7-hydroxylase (P-450coh). Unlike type I P-450$_{15\alpha}$, the other member within the mouse steroid 15α-hydroxylase gene family, type II catalyzed little steroid 15α-hydroxylase activity, yet structurally there were only 11 substitutions between type I and type II P-450$_{15\alpha}$s within their 494 amino acid residues (Lindberg et al., 1989), and the N-terminal sequence (21 residues) of P-450coh was identical with that of both P-450$_{15\alpha}$s. Induction by pyrazole of coumarin 7-hydroxylase activity correlated well with the increase of type II P-450$_{15\alpha}$ mRNA in 129/J male and female mice. Pyrazole, on the other hand, was less in males or not effective in females in inducing the 15α-hydroxylase activity and type I P-450$_{15\alpha}$ mRNA. Expression of type I and II in COS-1 cells revealed that the latter catalyzed coumarin 7-hydroxylase activity at 10 to ~14 pmol min^{-1} (mg of cellular protein)$^{-1}$. The former, on the other hand, had a high testosterone 15α-hydroxylase but little coumarin 7-hydroxylase activity. It was concluded, therefore, that type II P-450$_{15\alpha}$ is the mouse coumarin 7-hydroxylase. Identification of type II as the P-450 specific to coumarin 7-hydroxylase activity and characterization of its cDNA and gene, therefore, were significant advances toward understanding the basis of genetic regulation of this activity in mice (known as Coh locus).

Our laboratories have been investigating mouse coumarin 7-hydroxylase (P-450coh) and steroid 15α-hydroxylase (P-450$_{15\alpha}$) in liver microsomes for the past several years.

Wood and Conney (1974) and Wood and Taylor (1978) first discovered that basal and phenobarbital-induced coumarin 7-hydroxylase activity levels were regulated by the Coh locus in inbred mice and localized the locus to mouse chromosome 7. In addition to mice, this activity was present ubiquitously in rabbits, guinea pigs, hamsters, pigs, and humans, whereas rats contained little activity (Kaipainen et al., 1985; Pelkonen et al., 1985). Furthermore, an individual variation of the activity was found in the human population (Pelkonen et al., 1985; Raunio et al., 1988). Lang and his associates (Kuopio's group) found that pyrazole strongly induced coumarin 7-hydroxylase activity in DBA/2J mice (Juvonen et al., 1985). The group subsequently purified P-450coh from the drug-treated mice by using the high-spin spectrum of the P-450–coumarin complex as the basis for the selection of fractions from columns. P-450coh appeared to be the major enzyme specific to coumarin 7-hydroxylase activity in mouse liver microsomes (Juvonen et al., 1988).

P-450$_{15\alpha}$ was characterized as steroid 15α-hydroxylase by Negishi and his associates at the National Institute of Environmental Health Sciences (NIEHS); specific testosterone 15α-hydroxylase activity in fractions was used as the selection marker during the column chromatography to purify this P-450 from 129/J female mice (Harada & Negishi, 1984). P-450$_{15\alpha}$ was female specific in 129/J liver and catalyzed specifically 15α-hydroxylation of 3-keto Δ^4-steroids such as testosterone, progesterone, and androstenedione (Burkhart et al., 1985; Harada & Negishi, 1988). The cDNA cloning for P-450$_{15\alpha}$ revealed that in the steroid 15α-hydroxylase family there are two highly homologous type I and II P-450$_{15\alpha}$s, which have only 11 substitutions between them in their 494 amino acid residues (Squires & Negishi, 1988; Lindberg et al., 1989). We also determined the structures of genes 15αOH-1 and -2 which encoded type I and II, respectively (Lindberg et al., 1989). Separate expression of each P-450$_{15\alpha}$ in COS-1 cells revealed that in spite of such a high homology the catalytic activity of the two cytochromes differed (Lindberg et al., 1989); type I exhibited a high 15α-hydroxylation activity of the 3-keto Δ^4-steroids. Type II, however, catalyzed little activity toward these steroids. Thus, we concluded 15αOH-1-encoded type I to be the major steroid 15α-hydroxylase in mice, yet an activity specific to type II remained unknown at that time. In this paper, we report that type II P-450$_{15\alpha}$ is the mouse coumarin 7-hydroxylase.

Experimental Procedures

Animals. Male and female 2-month-old 129/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Pyrazole (200 mg/kg), which was dissolved in 0.9% NaCl solution, was injected intraperitoneally daily for three consecutive days.

Materials. [4-^{14}C]Testosterone (50 mCi/mmol) was purchased from Du Pont NEN Products, and coumarin and 7-hydroxycoumarin were from Aldrich Chemical Co. (Milwaukee, WI). BRL provided cDNA synthesis kits; Collaborative Research provided oligo(dT)–cellulose (type II). Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Gibco Laboratories (Long Island, NY); nitrocellulose and Nylon paper (Nytran) were from Schleicher & Schuell (Keene, NH). Anti-P-450$_{15\alpha}$ (Harada & Negishi, 1984) and purified P-450coh and its antibody (Juvonen et al., 1988) were prepared as previously described. NADPH–cytochrome P-450 reductase was purified from phenobarbital-

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1 21
M-L-T-S-G-L-L-L-V-A-A-V-A-F-L-S-V-L-V-L-M

FIGURE 1: N-Terminal sequences of type I and II P-$450_{15\alpha}$s and P-450coh are identical. The first 21 amino acid residues of type I and II P-$450_{15\alpha}$s (Squires & Negishi, 1988; Lindberg et al., 1989) are shown here, which are identical with those of P-450coh (Lang et al., 1989).

treated rat liver microsomes by the Yasukochi and Masters' method (1976).

Preparation of Liver Microsomes and Poly(A+) RNA. Livers from five mice were pooled for each group and then minced and separated into two parts. One part was used to isolate microsomes as described previously (Harada & Negishi, 1984), while poly(A+) RNA was prepared from the other part by extraction with guanidine hydrochloride (Cox, 1968) and subsequently by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972).

Expression of cDNAs in COS-1 Cells. The cDNAs p15α-16 and -2, which encoded type I and II P-$450_{15\alpha}$, respectively (Squires & Negishi, 1988; Lindberg et al., 1989), were ligated to Okayama–Berg's expression vector pcD and then transfected into COS-1 cells (ATCC, Rockville, MD). COS-1 cells were treated with DEAE-dextran and then transfected with a recombinant plasmid DNA according to the Sompayac and Donna (1981) method. After incubation with chloroquine (Luthman & Magnusson, 1983), the transfected cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 72 h at 37 °C. Finally, the cells were homogenated and used for measurement of coumarin 7-hydroxylase and testosterone 15α-hydroxylase activities. More details of the transfection were described previously (Lindberg et al., 1989).

Analytical Methods. Slot-blot hybridization of liver poly-(A^+) RNA with nick-translated ^{32}P-labeled p15α-16, a type I P-$450_{15\alpha}$ cDNA, was performed by the previously used method (Noshiro et al., 1986). Ratios of type I and II P-$450_{15\alpha}$ mRNAs in liver poly(A+) RNA were determined by the method described previously (Squires & Negishi, 1988). We performed Western blot by the method of Domin et al. (1984). The assay method of coumarin 7-hydroxylase activity was described previously (Kaipainen et al., 1985). Testosterone 15α-hydroxylase activity was measured according to Harada and Negishi (1984). The Bradford method (1976) was used to determine protein concentration.

RESULTS

Characteristics of Purified P-450coh. The N-terminal sequence (21 amino acids) of purified P-450coh, shown in Figure 1, was identical with the sequence corresponding to type I and II P-$450_{15\alpha}$s. In fact, the 42 residues from the type I N-terminus were identical with those of type II (Lindberg et al., 1989). Purified P-450coh catalyzed testosterone 15α-hydroxylase activity at 8.0 nmol min^{-1} (nmol of P-450)$^{-1}$, a level approximately $^1/_{10}$ that of purified P-$450_{15\alpha}$ (Harada & Negishi, 1984). Both anti-P-450coh and P-$450_{15\alpha}$ inhibited completely both coumarin 7-hydroxylase and testosterone 15α-hydroxylase activities of purified P-450coh in a reconstitution system (data not shown). P-450coh, therefore, was indistinguishable from type I and II P-$450_{15\alpha}$s; the only difference was that the 15α-hydroxylase activity was about $^1/_{10}$ of the P-$450_{15\alpha}$ activity.

Differential Effects of Pyrazole Treatment on Coumarin 7-Hydroxylase and Testosterone 15α-Hydroxylase Activities in 129/J Mice. We examined the effect of pyrazole treatment as an inducer on testosterone 15α-hydroxylase activity in 129/J mice (Table I), on the basis of the previous finding that the chemical induced coumarin 7-hydroxylase activity in liver microsomes of DBA/2 mice (Juvonen et al., 1988). For this purpose, the activities inhibited by an anti-P-$450_{15\alpha}$ were determined as specific to P-$450_{15\alpha}$- and/or P-450coh-dependent hydroxylation in microsomes. The use of anti-P-$450_{15\alpha}$ was necessary for these specific activities, since a previous work indicated the presence of a P-450 other than P-$450_{15\alpha}$, which catalyzed testosterone 15α-hydroxylation in mouse liver microsomes (Burkhart et al., 1985). Both activities were 6–9 times higher in untreated control females than in male 129/J mice. This was consistent with the previous results that female liver contained at least 6 times more P-$450_{15\alpha}$ mRNA (Burkhart et al., 1985). As expected, coumarin 7-hydroxylase activity was increased more than 20 times in the drug-treated male and 6 times in the female 129/J mice. Pyrazole induced testosterone 15α-hydroxylase activity in males only 6 times higher; the activity levels in female liver, however, were not significantly changed. Although the N-terminal protein sequences and immunochemistry were not able to distinguish P-450coh from either type I or type II P-$450_{15\alpha}$, it appeared that the activities associated with each cytochrome responded differently to pyrazole treatment in 129/J mice.

Table I: Effect of Pyrazole Treatment on Testosterone 15α-Hydroxylase and Coumarin 7-Hydroxylase Activities on 129/J Liver Microsomes[a]

	activity [pmol min^{-1} (mg of protein)$^{-1}$]			
	coumarin		testosterone	
	control	pyrazole	control	pyrazole
male	180	4230	110	640
female	1080	6750	920	1090

[a] Liver microsomes were prepared from untreated control and pyrazole-treated male and female 129/J mice (five mice for each group). Testosterone 15α-hydroxylase and coumarin 7-hydroxylase activities were measured by the methods described previously (Juvonen et al., 1985; Harada & Negishi, 1984). The values were averaged from two separate assays and expressed as the activities inhibited by anti-P-$450_{15\alpha}$. The amount of antibody which used for inhibition was enough to inhibit nearly 100% of P-$450_{15\alpha}$ and P-450coh-dependent activities.

Differential Induction of Type I and II P-450 mRNAs by Pyrazole. Slot-blot hybridization of liver poly(A+) RNAs with ^{32}P-labeled p15α-16 revealed a dramatic increase in the hybridizable mRNA in pyrazole-treated 129/J male mice. Further, a small (3–4-fold) mRNA increase was seen in drug-treated female mice (Figure 2a). Because of a high nucleotide homology between type I and type II P-$450_{15\alpha}$ mRNAs, the slot-blot hybridizations measured the mRNA contents as a sum of two P-$450_{15\alpha}$s. In order to examine whether type I and II mRNA levels were affected differently by pyrazole treatment as the hydroxylase activities revealed, we measured ratios of both mRNAs in liver poly(A+) RNAs from untreated control and drug-treated 129/J males and females. As shown in Figure 2b, pyrazole massively induced type II mRNA in both sexes, while type I mRNA was induced slightly in the male only up to the levels seen in untreated control females. In female liver, however, type I mRNA contents were not changed by the drug treatment. The results indicated that the type II increase correlated well with the induction of coumarin 7-hydroxylase and type I with testosterone 7-hydroxylase activities.

Expression of Type I and II cDNAs in COS-1 Cells. Both p15α-16 and p15α-2 cDNAs, which encoded type I and II P-$450_{15\alpha}$s, respectively, were ligated to Okayama–Berg's expression vector pcD and then transfected into COS-1 cells. Homogenates prepared from the transfected cells were used as enzyme sources to determine whether they catalyzed coumarin 7-hydroxylase activity.

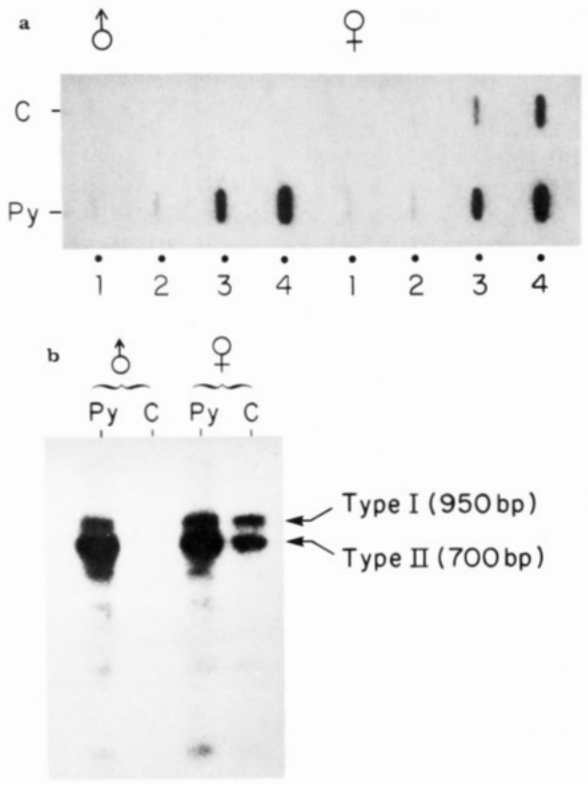

FIGURE 2: Differential induction by pyrazole of type I and II mRNAs in 129/J mice. (a) Slot-blot hybridization of liver poly(A+) RNA: Various amounts of poly(A+) RNA were applied on nitrocellulose paper, hybridized with nick-translated ^{32}P-labeled p15α-16, washed, and exposed to X-ray films. Slots 1–4 contained 0.03, 0.1, 0.3, and 1.0 μg of poly(A+) RNA, respectively. Py denotes poly(A+) RNA from pyrazole-treated mice; C denotes untreated control mice. (b) Effect of pyrazole treatment on type I and II mRNAs: From each liver poly(A+) RNA, double-stranded cDNAs (5 μg each) were synthesized, digested with *Cla*1 and *Pst*1, electrophoresed on agarose gel, and then transferred to Nytran paper. The Nytran paper was hybridized with the ^{32}P-labeled *Cla*1–*Cla*1 fragment from p15α-16. Since the two *Cla*1 sites are common, the same 950-bp fragment is generated from both types of cDNA. A unique *Pst*1 site cut type II's *Cla*1–*Cla*1 into two fragments whose sizes were 700 and 250 bp. The intensities of bands at 950 and 700 bp, therefore, represent the relative amounts of type I and II mRNAs in poly(A+) RNA samples, respectively.

Time-dependent increases of the activity in the homogenates prepared from type I or type II transfected COS-1 cells are shown in Figure 3. Type II dependent formation of 7-hydroxycoumarin was linearly increased for at least 45 min under the assay conditions. Its specific activity was 14 pmol min^{-1} (mg of protein)$^{-1}$, about $^{1}/_{100}$ of the activity in liver microsomes from 129/J female mice. Furthermore, the activity was inhibited completely by anti-P-450$_{15\alpha}$ (not shown). Type I, on the contrary, had no detectable coumarin 7-hydroxylase activity. As described in a previous report (Lindberg et al., 1989), the Western blot with anti-P-450$_{15\alpha}$ indicated that there were no significant differences in the amounts of expressed type I and II P-450$_{15\alpha}$s in the transfected cell homogenates.

Type I P-450$_{15\alpha}$ was identified as the steroid 15α-hydroxylase and possessed a high 15α-hydroxylation activity of 3-keto Δ^4-steroids such as testosterone, progesterone, and androstenedione (Lindberg et al., 1989). Although type I P-450$_{15\alpha}$ did not catalyze coumarin 7-hydroxylase activity, it was still possible that coumarin interacts somehow with this P-450. To test this possibility, we examined whether coumarin affected type I's testosterone 15α-hydroxylase activity. As shown in Figure 4, coumarin inhibited 90% of the 15α-hydroxylase activity at the concentration (250 μM) of testosterone used as substrate (Figure 5). The double-reciprocal plots with and without the presence of coumarin indicated that the compound is a noncompetitive inhibitor.

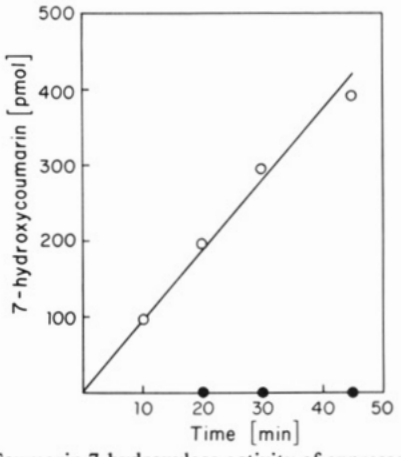

FIGURE 3: Coumarin 7-hydroxylase activity of expressed type I and type II P-450$_{15\alpha}$s in COS-1 cell homogenates. Harvested COS-1 cells from 32 plates (10-cm diameter) were homogenated in 1 mL of 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM KCl, 1 mM EDTA, and 5 μg/mL each of leupeptin and pepstatin, which were used as the enzyme source for each time point. The reaction mixture consisted of 25 mM potassium phosphate buffer, pH 7.4, 2.5 mM $MgCl_2$, 50 μM coumarin, the cell homogenate (100 μL or 760 μg of protein), and 750 μM NADPH in a 500-μL final volume. After incubation at 37 °C at the time intervals specified on the figure, the reaction was stopped by addition of 500 μL of 6% trichloroacetic acid. The formation of 7-hydroxycoumarin was measured by its fluorescence in alkaline solution as described previously (Kaipainen et al., 1985). The homogenates from mock-transfected cells exhibited no detectable coumarin 7-hydroxylase and testosterone 15α-hydroxylase activities. Type I (●); type II (○).

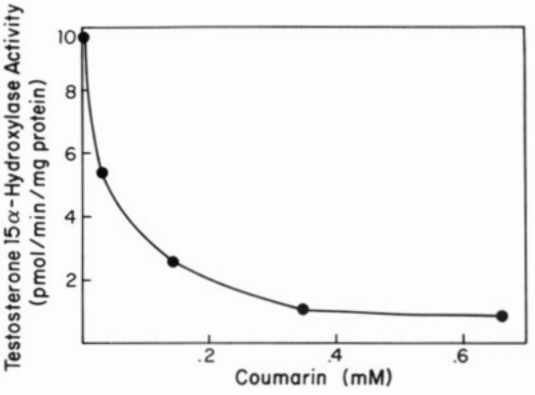

FIGURE 4: Inhibition by coumarin of type I dependent testosterone 15α-hydroxylase activity. Type I transfected COS-1 cell homogenates (250 μg of protein) were incubated with various amounts of coumarin for 1 min at 37 °C prior to the initiation of the enzyme reaction by addition of NADPH. All other reaction conditions were the same as those described previously for testosterone 15α-hydroxylase assay (Harada & Negishi, 1984).

Discussion

In this paper, we provided the evidence that type II P-450$_{15\alpha}$ is the coumarin 7-hydroxylase (P-450coh) in mice. Gene 15αOH-2, which was characterized to encode type II, therefore, is identified as the coumarin 7-hydroxylase gene, which consisted of nine exons with approximately 8 kbp of size (Lindberg et al., 1989).

The steroid 15α-hydroxylase gene family consists of two genes, 15αOH-1 and -2, which have duplicated approximately 5 million years ago (Lindberg et al., 1989). Type I and II P-450$_{15\alpha}$s, which are encoded by 15αOH-1 and -2, respectively,

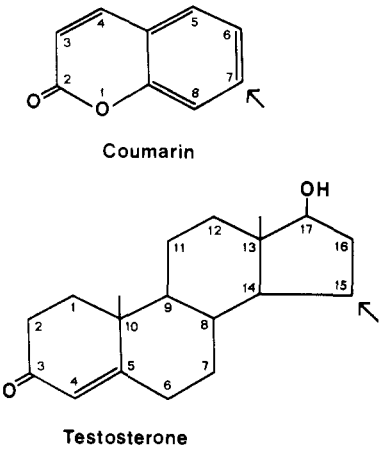

FIGURE 5: Structures of coumarin and testosterone. Arrows indicate the positions to be hydroxylated.

share 98.3% amino acid sequence homology (Squires & Negishi, 1988; Lindberg et al., 1989) and are expressed specifically in female 129/J mouse liver (Squires & Negishi, 1988). Despite these similarities between the cytochromes, only type I catalyzes specifically 15α-hydroxylation of testosterone, progesterone, and androstenedione. We concluded previously, therefore, that type I is the steroid 15α-hydroxylase (Lindberg et al., 1989). Since only type II P-450$_{15\alpha}$ catalyzed 7-hydroxylation of coumarin, we now identify type II as the coumarin 7-hydroxylase. This striking result indicates that N-terminal protein sequences are not sufficient criteria to identify cDNA encoding a P-450 specific to any given enzymatic activity. As shown in Figure 5, testosterone's Δ^4 3-keto structure is reminiscent of coumarin's Δ^3 2-keto structure. As the substrate specificity of type I indicates that the Δ^4 3-keto structure seems to be important for steroid binding to the 15α-hydroxylase (Harada & Negishi, 1988; Lindberg et al., 1989), the inhibition of type I dependent testosterone 15α-hydroxylase activity by coumarin suggests that its A-ring with Δ^3 2-keto structure works as a binding site to the 7-hydroxylase.

Basal and phenobarbital-induced coumarin 7-hydroxylase activity levels are regulated by the Coh locus on chromosome 7 in inbred mice (Wood & Conney, 1974; Wood & Taylor, 1978). Recently, Burkhart and Negishi (unpublished results) found 100% concordance between the steroid 15α-hydroxylase gene family and the Coh locus in nine strains of 9XA recombinant inbred mice (descended from A/HeJ and 129/sv-sl). Wood proposed that the Coh locus encodes coumarin 7-hydroxylase itself with the structural mutation, which results in the phenotypically different enzymes with a high activity in strains such as DBA/2J and 129/J (Coh^h homozygote), and a low activity in ARK/J (Coh^l homozygote), respectively (Wood, 1978). Kaipainen et al. (1985) purified P-450coh from DBA/2J and AKR/J mice and supported the previous conclusion. It is possible, therefore, that the 15αOH-2, type II P-450$_{15\alpha}$, gene is the Coh locus. Later, Lang et al. (1989) found that the pyrazole-induced levels of P-450coh protein were also regulated by the Coh locus. This finding, however, does not necessarily change Wood's earlier conclusion, although a possibility remains that pyrazole regulates 15αOH-2's transcription differently between DBA/2J and AKR/J mice.

The regulation by pyrazole is again surprisingly specific; coumarin 7-hydroxylase activity is induced in both sexes, whereas steroid 15α-hydroxylase activity is increased slightly in males but is not affected in females. These inductions are controlled pretranslationally. Since genes 15αOH-1 and -2 have such similar structures (Lindberg et al., 1989), it will be interesting to know how pyrazole regulates these genes differentially.

In conclusion, the steroid 15α-hydroxylase gene family consists of steroid 15α-hydroxylase (type I P-450$_{15\alpha}$) and coumarin 7-hydroxylase (P-450coh or type II P-450$_{15\alpha}$). Gene 15αOH-2, which encodes the coumarin 7-hydroxylase, might be the Coh locus itself. This gene family provides an excellent system to study sex-specific gene regulation by hormones, as well as by drugs such as pyrazole. Type I and II P-450$_{15\alpha}$s will be an effective, simple model for studying the P-450's reaction mechanisms such as substrate specificity, activity–structure relationship, and evolution. We now have the tools to uncover the Coh locus and will be able to investigate the genetic and molecular bases of coumarin 7-hydroxylase's polymorphism in the human population.

Registry No. P-450coh, 39401-02-0; P-450$_{15\alpha}$, 70699-33-1; P-450, 9035-51-2; pyrazole, 58-22-0; testosterone, 58-22-0; coumarin, 91-64-5.

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