Biochemical Pharmacology, Vol. 31, No. 11, pp. 1993–1998, 1982.
Printed in Great Britain.

0006-2952/82/111993-06 $03.00/0

INTERACTION OF THE SUBSTRATE ANALOGUE OF CYTOCHROME P-450 AND MIXED FUNCTION OXIDASES

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(*Received* 8 *October* 1980; *accepted* 10 *December* 1981)

Abstract—The interaction of a spin labeled compound carrying an alkylating group 4-(3-iodo-2-oxopropylidene)-2,2,3,5,5-pentamethylimidozolydene-1-oxyl (RJ) and capable of binding covalently to mixed function oxidases (MFO) was studied. Measurements of the difference spectrum of cytochrome P-450 demonstrated that RJ induces spectral changes characteristic of type I substrates (λ_{max} = 403 nm; λ_{min} = 418 nm). The spectral binding constant (K_s) was 66 μM as determined from the difference spectrum. RJ inhibited the microsomal oxidation of substrates of cytochrome P-450 (aniline, aminopyrine and benzo [*a*]pyrene). This inhibition was shown not to be associated with the conversion of cytochrome P-450 to cytochrome P-420, or with the suppression of the activities of NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase. Thus, evidence was obtained for the possible interaction of RJ with cytochrome P-450. RJ, injected to rats (5 mg/100 g body wt, i.p.), inhibited the hydroxylation of benzo[a] pyrene, a type I substrate, (21%) and aniline, a type II substrate, (40%) in the microsomes from their livers. The presence of a paramagnetic center in RJ made it possible to study its interaction with microsomes. The electron paramagnetic resonance (EPR) spectrum of RJ was recorded in the rat liver microsomal fraction after *in vivo* administration of RJ.

In rats treated with RJ (5 mg/100 g), hexobarbital sleeping time was prolonged 1.5-fold. Alkylating analogs of substrates of cytochrome P-450 are suggested as agents for structural studies of the active center of cytochrome P-450 and the development of efficient inhibitors of reactions catalyzed by this enzyme.

The terminal component of MFO is a hemoprotein cytochrome P-450, forming complexes with the compounds oxidized by it [1, 2]. Because cytochrome P-450 oxidizes organic compounds with different structures, it appeared of interest to obtain more precise data concerning the structure of its active center. Photoaffinic and alkylating inhibitors have been used to modify cytochrome $P\text{-}450_{cam}$ and different species of cytochrome P-450 from rabbit liver [3, 4]. Degradation of the modified proteins by the BrCN technique has made it possible to determine the amino acid composition of the peptide fragment covalently binding with the inhibitor. Another application of affinity modification was the use of alkylating analogs of the substrates containing a spin label [5]. It enabled us to measure the distance between the nitroxide group of radical RJ (covalently bound to cytochrome P-450) and Fe^{3+} in the active center of cytochrome P-450. The inhibitory effect of RJ observed suggested that we take advantage of this property not only *in vitro* but also *in vivo*.

In the present work we investigated the effects of RJ on the activities of the microsomal electron transport enzymes and the capacity of this radical to inhibit the enzymatic oxidation of cytochrome P-450 substrates type I (aminopyrine, benzo[*a*]pyrene) and type II (aniline). Experiments were also conducted with the aim to determine whether hexobarbital sleeping time is prolonged in RJ-treated rats.

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MATERIALS AND METHODS

Chemicals. NADPH (Reanal): the concentration of NADPH was determined by u.v. absorption using $\xi_{340} = 6.22 \times 10^3 M^{-1}cm^{-1}$ (0.1 M Tris–HCl buffer, pH 7.6). Aniline (Soyuzkhimreaktiv, Moscow, U.S.S.R.) was purified by vacuum distillation; aminopyrine (1-phenyl-2,3-dimethyl-4-dimethylaminopyrasolon-5) (Soyuzkhemreaktiv, Moscow, U.S.S.R.), 3,4-benzo[*a*]pyrene (Serva), [6-^{3}H]benzo[*a*]pyrene (28 Ci/mmole) (Radiochemical Centre, Amersham, U.K.), hexobarbital (a commercial preparation) were used without additional purification; radical 4-(3-iodo-2-oxopropylidene)-2,2,3,5,5-pentamethylimidazolydene-1-oxyl (RJ) [6] was kindly provided by Prof. Volodarskii (Institute of Organic Chemistry, Novosibirsk) (Fig. 1).

Preparation of microsomes. Livers were obtained from male Wistar rats (120–140 g). Microsomes were prepared by the standard procedure [7]; protein content was determined by Lowry's method [8] using bovine serum albumin (BSA) as a standard. The concentration of cytochrome P-450 was determined as described by Omura and Sato [9] with the extinction coefficient $\xi_{450} = 91 \times 10^3 M^{-1}cm^{-1}$.

Spectral interactions. For the determination of spectral interactions, the microsomal suspension was diluted with phosphate buffer to a final protein concentration of about 1.5–2 mg/ml. All spectra were measured at room temperature in a 1 cm cell of a Hitachi-556 recording spectrophotometer. Baselines were traced. Difference spectra were obtained by supplementing a solution of RJ in acetonitrile to one

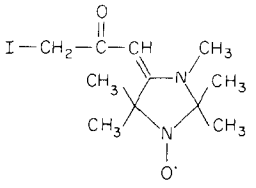

Fig. 1. Structural formula of the inhibitory RJ.

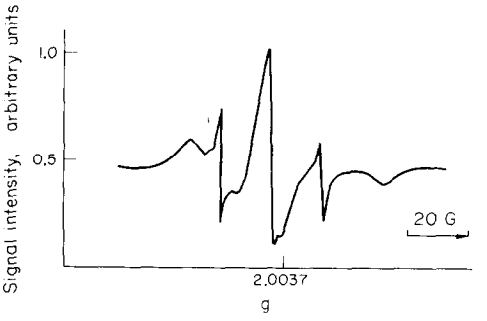

Fig. 2. The EPR spectrum of a RJ radical bound to BSA. For spin-labelling of bovine serum albumin the protein was incubated with RJ at a molar ratio of approx. 1 to 5 at 15° for 30 min. The mixture was then thoroughly dialyzed against 100 mM Tris–HCl buffer, pH 7.6, the number of spin labels attached to the protein was some 1.0 per molecule. Spectrometer settings were: temperature 25°, modulation amplitude 2 G, scan range 100 G, receiver gain 2.5×10^3, power 50 mV, frequency 9.45 GHz, time constant 1 sec.

of the cuvettes; an equal volume of the solvent was added to the reference cuvette. The extent of the spectral change was measured from the difference between the wavelengths of minimum and maximum absorption. The spectral binding constant (K_s) was determined by plotting the reciprocal of the changes in absorbance vs the reciprocal of RJ concentrations [10].

Electron paramagnetic resonance (EPR) spectra were recorded with the E-3 spectrometer (Varian) using a flat ampule at 25°. Before recording the spectral changes, $K_3Fe(CN)_6$ was added to the sample cuvette to oxidize the partially reduced RJ. The concentration of this supplement (5×10^{-4}M) did not produce any broadening of the signal.

In vivo *studies*. Rats received i.p. an aqueous sonicated suspension of RJ at a dose of 5 mg/100 g body wt. A dose of RJ exceeding 10 mg/100 g body wt. was found to be toxic. Rats were injected i.p. with hexobarbital (10 mg/100 g body wt) in experiments designed to determine its sleeping time. The sleeping time was expressed as the time during which the animals lay immobilized on one side.

In experiments conducted to study the effect of RJ on hexobarbital sleeping time, rats received i.p. RJ 15 or 30 min before the administration of hexobarbital. In subsequent *in vivo* experiments rats were injected i.p. with RJ and decapitated 60 min after the injection; the livers were removed and microsomes were prepared from them. The concentration of RJ was 50 mg/100 g body wt. and this dose did not produce any significant changes in the behaviour of rats during 60 min between the moment of injection and decapitation. The microsomes from these rats were used for hydroxylation. The reaction of hydroxylation of aniline, benzo[*a*]pyrene and aminopyrine was determined by estimating the amounts of *p*-aminophenol [11], 3-hydroxybenzo[*a*]pyrene [12] and formaldehyde [13] formed.

Estimates of the effect of RJ on NADPH–cytochrome P-450 reductase were based on the accumulation rate of reduced cytochrome P-450 in the presence of CO [14]. The effect of RJ on NADPH–cytochrome *c* reductase was measured according to [15].

RESULTS AND DISCUSSION

It is well known that certain classes of halogen-containing compounds can alkylate SH, OH, NH_2 groups of protein [16]. In a previous paper, we presented tentative evidence for the capacity of RJ to covalently bind to microsomal cytochrome P-450 [5]. Inasmuch as alkylating spin label binds to SH group, we additionally tested the alkylating capacity of RJ by modifying BSA [17]. The results of the test are presented in Fig. 2. Judging by the EPR spectrum, RJ bind covalently to BSA. According to our previous experimental data RJ at low concentrations should inhibit the oxidation of type I and II cytochrome P-450 substrates [5]. The drawback of these experiments was that ethanol (used at a final concentration of 5% in the reaction mixture) is not the optimum solvent for RJ; in the microsomal system used it binds to cytochrome P-450 and thereby affects the oxidation of cytochrome P-450 substrates [18]. In the present work acetonitrile was utilized as a solvent because it has no strong inhibitory effect on hydroxylation reactions [19, 20]. At the concentration employed, acetonitrile (10 μl/ml) only slightly (~2%) inhibited the microsomal hydroxylation of

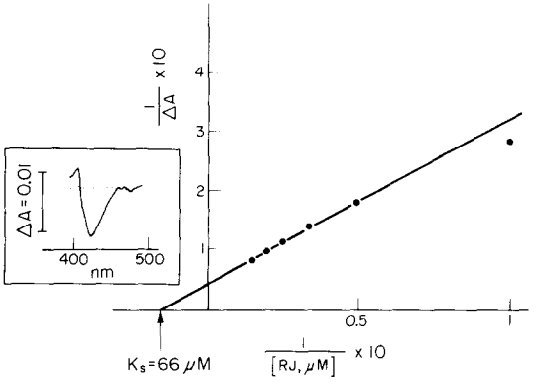

Fig. 3. Spectral changes induced by RJ with liver microsomes. The sample and the reference cuvettes contained 3 ml suspension of 4 mg of microsomal protein/ml in 0.1 M potassium phosphate buffer, pH 7.4. Aliquots of 10 μM RJ in acetonitrile were added to the sample cuvette to give the final concentration of 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, while equivalent aliquots of acetonitrile were added to the reference cuvette. The left-hand side of the figure shows spectral changes induced by 50 μM RJ addition (———), base line (· · · · · ·).

Table 1. Effect of RJ on the activities of NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase

System*	Reduction rate of cytochrome *c* (nmoles/mg protein/min)	Reduction rate of cytochrome P-450 (nmoles/min)
Complete system	75	1.6
Complete system + acetonitrile	75	1.78
Complete system + RJ	75	1.62

* The conditions of the inhibition are described in Materials and Methods.

the substrates. In all the experiments an equal volume of acetonitrile was supplemented to each reference sample. Figure 3 presents the spectral changes induced by RJ in microsomal cytochrome P-450. As seen in Fig. 3, the addition of this radical produces a difference spectrum with characteristic maxima and minima for the binding spectra of cytochrome P-450 with type I substrate (λ_{max} = 403 nm and λ_{min} = 418 nm). The spectral binding constant was about 66 μM.

The relation between MFO activity and the RJ concentration during the oxidation of different substrates of cytochrome P-450 is depicted in Fig. 4. It is clear that RJ inhibits more efficiently the oxidation of type II substrate than that of type I substrate. This is in agreement with our previous data [5]. The high alkylating capacity of RJ and the susceptibility of many microsomal enzymes to its modifying effects prompted us to perform more detailed investigations. The results of studies of the influence of RJ on the activities of the microsomal electron transport enzymes are summarized in Table 1. RJ has no influence on NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase and hence the inhibition observed (Fig. 4) is also unrelated to an RJ effect on the activity of these enzymes.

Determination of the effect of different RJ concentrations on cytochrome P-450 content demonstrated that in the concentration range of 10^{-7}–10^{-3} M cytochrome P-450 amounts did not change, i.e. cytochrome P-450 was not converted to its enzymatically inactive form, cytochrome P-420.

In the reported study RJ was incubated with rat liver microsomes, the reaction mixture was treated with a 4% solution of sodium cholate and separated into lipid and protein components by passage through a Biogel P-30 column [5]. As a result, RJ was present in the protein fraction only. The distance between the microsomal protein and Fe^{3+} in the active center of cytochrome P-450 was ~11Å as determined by the EPR spectra. From the results of the previous [5] and the present study it may be concluded that the inhibitory effect of RJ (Fig. 4) results from the covalent binding of RJ with the

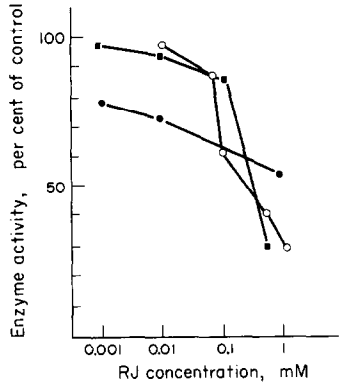

Fig. 4. Effects of varying concentrations of the inhibitor RJ on the yield of *p*-aminophenol (—○—), 3-hydroxybenzo[*a*]pyrene (—●—), and formaldehyde (—■—) during microsomal hydroxylation. The sample contained per 1 ml: microsomal protein 1.5 mg/ml (cytochrome P-450 3×10^{-6} M), aniline (aminopyrine) 10^{-3} M, NADPH 3×10^{-3} M, Tris–HCl buffer 0.1 M (pH 7.6), $MgCl_2$ 5×10^{-3} M, and RJ was added in 10 μl of acetonitrile. Incubation was started after a 10 min preincubation period and took place in a metabolic shaker at 37°. Incubation lasted 30 min [11, 13]. In the case of benzo[*a*]pyrene, the sample contained per 1 ml: NADP 10^{-3} M, isocitrate 6×10^{-3} M, isocitrate-dehydrogenase 0.36 units, benzo[*a*]pyrene 8×10^{-5} M, [^{3}H]benzo[*a*]pyrene 0.5 μCi, $MgCl_2$ 5 mM, Tris–HCl buffer 0.05 M (pH 7.5); after a 10 min preincubation period the mixture was incubated for 15 min. The subsequent operations were as described in [12].

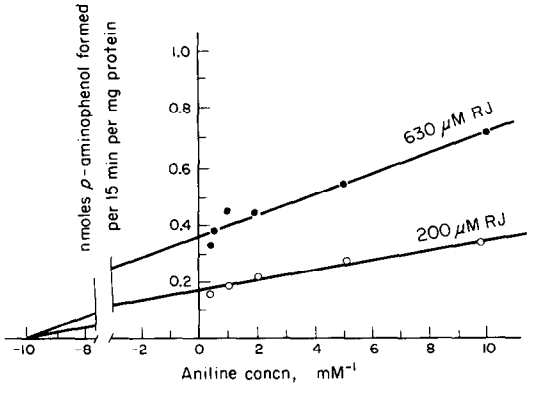

Fig. 5. Lineweaver–Burk plot of the inhibition of aniline hydroxylation by RJ. The incubation mixture contained microsomal fraction (4 mg of protein/ml, P-450 = 2×10^{-6} M) RJ and 100 mM Tris–HCl buffer, pH 7.6 in a total volume of 1 ml.

Table 2. Protective effect of aniline against the inactivation of cytochrome P-450 by RJ

Reaction conditions	Yield of product of the microsomal hydroxylation of aniline (%)
Complete reaction*	100
Complete reaction + RJ†	37
Complete reaction + RJ‡	69

* The reaction mixture contained: Tris–HCl, 0.1 M (pH 7.6); $MgCl_2$, 5×10^{-3} M; NADPH, 10^{-3} M; microsomes (cytochrome P-450, 1.2×10^{-6} M); RJ, 5×10^{-4} M.

† RJ was incubated with microsomes for 15 min and the other components of the reaction were added to the incubation mixture.

‡ RJ was incubated with microsomes for 15 min in the presence of substrate aniline and then the other components of the reaction were added to the incubation mixture.

active center of cytochrome P-450. To verify this conclusion, we conducted experiments to confirm whether the substrate of cytochrome P-450 has indeed a protective effect against its inactivation by RJ. The pertinent experimental data are given in Table 2. The data indicate that in the presence of aniline the inactivation rate of the MFO decreases appreciably. In our previous work [5] we were unable to detect the protective effect of cytochrome P-450 inactivation by RJ because of the strong inhibition of the solvent ethanol. Taken together, the data support the concept of high affinity of RJ to cytochrome P-450. Figure 5 is a Lineweaver–Burk plot representing the relation between the aniline oxidation rate and various RJ concentrations. It is evident from this figure that the kinetics curves follow a course characteristic of non-competitive inhibition [21]. It will be recalled that the kinetics curves of non-competitive inhibition type in the case of a covalently bound inhibitor call for cautious interpretation and a broader theoretical framework [21].*

One of the consequences of the inhibition of the oxidation of xenobiotics is the prolongation of the pharmacological effect of a number of drugs. Thus, the inhibition of MFO prolongs the soporific effect of hexobarbital [23, 24]. We employed the hexobarbital soporific test to investigate the *in vivo* possible inhibitory properties of RJ. It was found that hexobarbital sleeping time was 1.5-fold prolonged in RJ-treated rats (Table 3). A pertinent earlier finding was that the administration of the competitive inhibitor 17-α-hydroxydeoxycorticosterone (drug-S) 15 min before hexobarbital treatment ensured maximum prolongation of the sleeping time; when the drug-S was given 25 min before hexobarbital, the prolongation effect was much attenuated, possibly because the time elapsed was sufficient for hydroxylation of the inhibitor, which significantly decreased its affinity to cytochrome P-450 and promoted its transformation and excretion [23]. We also administered the inhibitor RJ to rats 15 or 30 min before hexobarbital injection. Hexobarbital sleeping was prolonged 1.5-fold irrespective of whether the inhibitor was given 15 or 30 min before the injection. (This is in contrast to the prolongation achieved by the drug-S.) This suggested that RJ, when injected i.p., inhibits the microsomal oxidation of hexobarbital, a substrate of cytochrome P-450, by binding

Table 3. Effect of RJ on hexobarbital sleeping time in Wistar rats

Treatment	Sleeping time* (min)
Control	54 ± 3 (42)
Treated with RJ 15 min before hexobarbital injection	80 ± 5 (30)
Treated with RJ 30 min before hexobarbital injection	76 ± 5 (22)

* Values in parentheses are the numbers of rats. The difference from controls were significant using Student's t test ($P < 0.01$). Results are expressed as means ± S.E.M. of hexobarbital sleeping times.

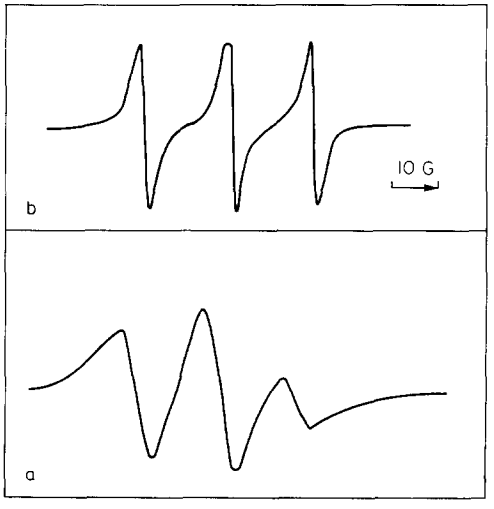

Fig. 6. EPR spectra of the RJ radical. (a) Microsomes from the livers of rats that received i.p. an aqueous solution of RJ at the dose of 50 mg/100 g body weight. Spectrometer settings were: temperature 25°, modulation amplitude 1 G, scan range 100 G, gain 1.25×10^6, power 100 mV, frequency 9.45 GHz, time constant 1 sec. (b) Microsomes with RJ radical added just before spectral registration. The sample contained: 40 mg/ml of microsomal protein. The concentration of RJ was 1.25×10^{-5} M. Spectrometer settings were: temperature 25°, modulation amplitude 1 G, scan range 100 G, gain 2.25×10^5, power 12.5 mV, frequency 9.45 GHz, time constant 1 sec.

* In the specific case of an inhibitor, which covalently binds to an enzyme, the kinetic parameters are determined by the procedure of Kitz and Wilson [22]

$$E + I \overset{K}{\rightleftarrows} E\text{–}I \overset{k}{\rightarrow} EZ \qquad (1)$$

where E denotes the enzyme, I is the inhibitor, E–I is the reversible enzyme–inhibitor complex and EZ is the inactive enzyme, the product of the modification; K is the dissociation constant of the E–I complex, and k the rate constant of the monomolecular conversion in the E–I complex. Based on the above formula (1) we have calculated the kinetic parameters for monooxygenase inhibition by RJ in the microsome from phenobarbital-induced and control rats. These quantitative estimates merit consideration in a separate publication.

Table 4. Effect of RJ on the enzymatic oxidation of aniline and benzo[*a*]pyrene of the liver microsomal fraction in Wistar rats*

Substrate	Groups of Wistar rats	
	Control	Experimental
Aniline	100%	60%†
Benzo[*a*]pyrene	100%	79%‡

* Male Wistar rats were injected i.p. with an aqueous suspension of RJ (50 mg/100 g body wt). Rats of control group received the same amount of the solvent. 60 min after RJ injection the microsomes were isolated from the livers of control and experimental animals as described in Materials and Methods.

† $P < 0.01$.

‡ $P > 0.01$.

covalently to it. To examine this possibility, we obtained a liver microsomal fraction from rats injected i.p. with RJ. A salient triplet with a_N = 16.2 *Qe* (Fig. 6(a)) indicates that RJ is present in the microsomal preparation. Figure 6(b) presents an EPR spectrum of RJ which was added to the reference sample immediately before recording the EPR.

Comparison of Fig. 6(a) and 6(b) shows that the mobility of radical RJ in the liver microsomal fraction from RJ-treated rats is lower than in the reference sample. The broadening of the signal is due to the covalent binding of RJ to the proteins of the microsomal fraction.

The result of the experiments with enzymatic oxidation of the cytochrome P-450 substrates by this microsomal fraction demonstrate that the hydroxylation rate of aniline and benzo[*a*]pyrene is decreased as compared with microsomes from the liver of untreated rats (Table 4). The oxidation of aniline was more inhibited than that of benzo[*a*]pyrene, which is in accordance with our *in vitro* observation (Fig. 4).

The data obtained on the effect of an alkylating substrate analogue of cytochrome P-450 upon MFO activities suggest that one can take advantage of affinity modification of cytochrome P-450 to inhibit the hydroxylation reactions accomplished *in vivo* by this enzyme.

It is noteworthy that the presence of a stable nitroxide radical in the inhibitor is without any influence on its ability to hinder the microsomal hydroxylation of cytochrome P-450 substrates. Supporting evidence was provided by experiments with inhibition of the enzymic hydroxylation of aniline by the nonradical analogue of RJ containing an $NOCH_3$-group instead of N—O· [5]. The data of this work, along with those reported earlier, are clear evidence for the capacity of RJ to bind covalently to the active center of cytochrome P-450. The use of substrate analogue containing spin labels for affinity modification of cytochrome P-450 may open up new avenues of knowledge in the application and interpretation of structural data concerning the active center cytochrome P-450 obtained by the EPR method. The obvious consequence of the inhibition of the monooxygenase activity of this enzyme is its binding to the active center of cytochrome P-450. We have observed this inhibition in our experiments with the oxidation of aminopyrine, benzo[*a*]pyrene, aniline and naphthaline [5]. The characteristic feature of this inhibition was that it was not associated with an inhibition of other microsomal activities of NADPH–cytochrome *c* reductase and NADPH–cytochrome P-450 reductase (see Table 1). This is in contrast to the observed inhibition of NADPH–cytochrome *c* reductase when a non-affinity alkylating compound was used [25]. The prolongation of hexobarbital sleeping time of rats (Table 3) and the decrease in monooxygenase activities in the microsomes from RJ-treated rats may also be explained by the covalent binding of RJ to protein. Admittedly, the RJ reagent we utilized is not the optimum inhibitor of oxidation reactions catalyzed by cytochrome P-450. Nevertheless, taking into account a large variety of substrates of cytochrome P-450 and to introduce chemically active groups into this substrate, we hope that there is a real basis for searching efficient inhibitors of monooxygenases needed for advances in pharmacology and medicine.

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