Journal of Chromatography, 532 (1990) 65–74
Biomedical Applications
Elsevier Science Publishers B.V , Amsterdam

CHROMBIO 5430

Enantiospecific assay for mammalian carbonyl reductase by liquid chromatography with fluorescence detection

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(First received February 12th, 1990; revised manuscript received May 28th, 1990)

ABSTRACT

A fluorogenic substrate with an unsymmetrical carbonyl for the sensitive assay of mammalian carbonyl reductase activities, 4-(6-methoxy-2-benzoxazolyl)acetophenone (I), has been prepared. The fluorescence quantum yield of I in acetonitrile is 0.12 at the emission maximum of 448 nm. The corresponding racemic alcohol produced by the chemical reduction of I, (±)-*sec*.-[4-(6-methoxy-2-benzoxazolyl)]phenethyl alcohol (II), exhibits *ca* three- to fourteen-fold higher fluorescence at a shorter wavelength emission maximum of 370 nm in conventional solvents. Each enantiomer of II is sufficiently resolved on a chiral cellulose high-performance liquid chromatographic column without derivatization and quantified with high reproducibility The detection limit for II is 20 fmol per injection at a signal-to-noise ratio of 3 The validity and applicability of I are evaluated with cytosols of mammalian tissues The optimal pH for metabolic reduction of I in rabbit liver cytosol preparations is 6.2 in the presence of NADPH. The metabolism is proved to be highly stereoselective. The resulting alcohol produced by mammalian tissue preparations, except rabbit kidney, is predominantly of the *S*-(−)-configuration.

INTRODUCTION

A large number of compounds of biological interest contain carbonyl moieties, and their reductive biotransformation is recognized as an important metabolic pathway not only in mammals [1–3] but also in other vertebrates, invertebrates and bacteria [4]. The physiological and biochemical natures of carbonyl reductases (EC 1.1.1.X) were not well clarified so far. However, extensive research has been reported recently, in which carbonyl reductases are characterized into several classes of isozymes according to their substrate specificity or inhibition studies [5–8]. Many substrates for this purpose are available, *e.g.* aliphatic, alicyclic and aromatic aldehydes and ketones, including endogenous ketosteroids or prostanoids [9–11]. The activity of carbonyl reductase has usually been assayed by monitoring the consecutive decrease of UV absorbance or fluorescence derived from reduced pyridine nucleotides, *e.g.* NADPH and NADH. However, this approach was disadvantageous because when such substrates or products have a strong chromogenicity they may often hinder the selectivity and reliability of the assay. Besides, it is impossible to explore the inherent stereochemical preference

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of enzymes against unsymmetrical carbonyls, *i.e.* which enantiomeric alcohol is predominantly produced [6,12].

This paper describes the synthesis of a new fluorogenic substrate for mammalian carbonyl reductases, 4-(6-methoxy-2-benzoxazolyl)acetophenone (I, Fig. 1), which allows the selective and sensitive assay of enzymes, including stereochemical preference. Compound I was further applied to evaluate the properties of carbonyl reductases in rabbit or rat tissues by linking with an enantiospecific high-performance liquid chromatographic (HPLC) method.

EXPERIMENTAL

Materials

All chemicals for synthesis or enzyme assay were of analytical-reagent grade unless noted otherwise. Reduced pyridine nucleotides (NADPH, NADH) were products of Sigma (St. Louis, MO, U S.A.). 3-Acetyl-7-(dimethylamino)coumarin (III), the internal standard for enantiomeric HPLC, was prepared from 4-(dimethylamino)salicylaldehyde and ethyl acetoacetate according to Knoevenagel condensation [13,14]. 2-Propanol and *n*-hexane for the chromatographic mobile phase were of HPLC grade and purchased from Wako (Osaka, Japan). Mammalian tissue preparations for use as enzyme sources were prepared as follows: male Albino rabbits (2.4–2.8 kg) or male Wistar–Imamichi rats (210–220 g), fasted overnight prior to the experiment, were killed by decapitation. Each liver, kidney, lung or intestinal mucosa was immediately excised and homogenized in four volumes of ice-chilled potassium phosphate buffer (50 m*M*, pH 7.4) containing 0.5 m*M* dithiothreitol. The cytosol fraction was obtained as a supernatant after centrifugation at 105 000 *g* for 1 h and subjected to the enzyme assay without further purification.

Synthesis of 4-(6-methoxy-2-benzoxazolyl)acetophenone (I)

To 9 g of ethyl 4-acetylbenzimidate hydrochloride salt were immediately added 200 ml of a methanol solution of 2-amino-5-methoxyphenol (5.5 g), which was

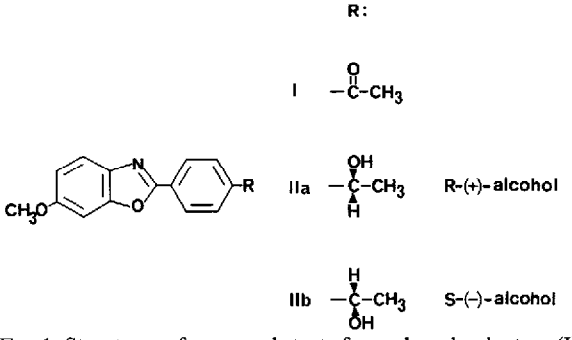

Fig 1. Structures of a new substrate for carbonyl reductase (I) and the corresponding enantiomeric alcohols (IIa and IIb)

prepared by catalytic hydrogenation of 2-nitro-5-methoxyphenol with PtO_2 as previously described [15]. The mixture was stirred at 60–70°C for 4 h. After evaporation *in vacuo*, the crude material was filtered and recrystallized from ethanol to give I as 8.16 g of fine orange– yellow crystals M.P., 167°C; yield, 76.4%, NMR($CDCl_3$), δ: 2.66 (3H, s, CH_3CO–), 3.89 (3H, s, CH_3O–), 6.9–8.4 (7H, m, aromatic H).

Synthesis of (±)-sec.-[4-(6-methoxy-2-benzoxazolyl)]phenethyl alcohol (II)

The racemic alcohol product, II, was prepared by the chemical reduction of I with sodium borohydride. To 8 g of I dissolved in 600 ml of methanol were added 360 mg of sodium borohydride at ambient temperature. After stirring for 2 h, the crude mixture was extracted with ethyl acetate and washed with 50 ml of water. The amber-coloured organic layer was dried over $MgSO_4$ and then subjected to silica gel column chromatography. The main fraction eluted with the mixture of dichloromethane and ethyl acetate (1:1, v/v) was evaporated to dryness to give II as 5.4 g of pale pink prisms. M.P., 127°C; yields, 66.9%, NMR($CDCl_3$), δ: 1 53 (3H, d, J=6.4 Hz, CH_3–), 2.30 (1H, bs, –OH), 3 87 (3H, s, CH_3O–), 4.95 (1H, q, J=6.4 Hz, –CH–), 6.9–8.3 (7H, m, aromatic).

Optical resolution of racemic alcohol product

The racemic alcohol, II, can be optically resolved by derivatization with (1*R*)-(−)-camphanic acid chloride as a chiral reagent to form its diastereomeric ester, IV. To 9 g of II, dissolved in 45 ml of anhydrous pyridine, were added 7.38 g of acid chloride with stirring for 1 h at ambient temperature. The reaction mixture was extracted with 200 ml of dichloromethane and evaporated *in vacuo* to give a pale pink oil. The diastereomeric ester was completely dissolved in 600 ml of methanol by heating on a bath and kept stationary overnight. The needles were collected by filtration, washed with a minimal amount of cold methanol and recrystallized six times from methanol to give 0.9 g of IVa as white needles. M.P., 171°C; $[\alpha]^{20}D$, +29.8° (*c* 0.30, $CHCl_3$); NMR($CDCl_3$), δ: 0.86 (3H, s, CH_3–C–CO–), 1.03 and 1.11 (each 3H, each s, $(CH_3)_2$–C–), 1.65 (3H, d, J=6.0 Hz, $\underline{CH_3}$–CH–O–), 1.7–2.7 (4H, m, methylene of camphanyl), 3.88 (3H, s, CH_3O–), 6.08 (1H, q, J=6.0 Hz, $CH_3\underline{CH}$–O–), 6.9–8.3 (7H, m, aromatic). The crystals were hydrolysed with 50 ml of 1.2 *M* NaOH in methanol followed by the extraction with ethyl acetate. The extract was dried over $MgSO_4$ and recrystallized from diethyl ether to give the *R*-(+)-enantiomer of II, IIa, as white needles (M.P., 109°C). The more soluble diastereomer, IVb, in supernatant was similarly given by condensation and repeated recrystallization from ice-cold methanol as 1.02 g of white needles. M.P., 153°C; $[\alpha]^{20}D$, −40.2° (*c* 0.47, $CHCl_3$), NMR($CDCl_3$), δ: 0.95 (3H, s, CH_3–C–CO–), 1.03 and 1.12 (each 3H, each s, $(CH_3)_2$–C–), 1.64 (3H, d, J=6.0 Hz, $\underline{CH_3}$–CH–O–), 1.7–2.7 (4H, m, methylene of camphanyl), 3.88 (3H, s, CH_3O–), 6.08 (1H, q, J=6.0 Hz, $CH_3\underline{CH}$–O–), 6.9–8.3 (7H, m, aromatic). Compound IVb was hydrolysed in a similar manner to obtain the *S*-(−)-

enantiomer of II, IIb (M.P. 108°C). The overall recovery on the resolution was 21.3% The molecular structures of the substrate, I, and the corresponding enantiomeric alcohols, IIa, and IIb, are shown in Fig. 1.

Instruments

A Model LC6A HPLC solvent-delivery and sample-processing system (Shimadzu, Kyoto, Japan), equipped with a Model F-1000 fluorescence detector (Hitachi, Tokyo, Japan), was used. UV absorption was measured with a Model 200-20 spectrophotometer (Hitachi). The fluorescence spectra of authentic derivatives, I and II, were measured on a Model RF-540 fluorescence spectrophotometer (Shimadzu, and their relative fluorescence quantum yields were calculated with quinine sulphate ($\Phi_f = 0.70$) in 0.05 *M* sulphuric acid, as the standard [16]. In order to assess the absolute configuration of each enantiomer, specific rotations were measured with a Model 243 polarimeter (Perkin-Elmer, Norwalk, CT, U.S.A.) by the comparison with a camphanyl ester of authentic *R*-(+)- or *S*-(−)-*sec.*-phenethyl alcohol.

Enzyme assay

The reductase activity was ordinarily assayed in an incubation mixture containing the substrate, I, dissolved in 0.1 ml of acetonitrile to give a final concentration of 500 μM, 200 μM NADPH, 0.1 ml of cytosol and 10 mg/ml bovine serum albumin in 0.1 *M* potassium phosphate buffer (pH 6.2). The final volume was 2.0 ml. The substrate concentration ranged between 0.5 μM and 1 m*M* for the enzyme kinetic study. The reaction was initiated by the addition of cytosol, and the mixture was incubated at 25°C under aerobic conditions. Each reaction was performed in duplicate with monitoring of the decrease of absorption at 340 nm. After incubation for 10 min, the reaction was terminated by the addition of 0.2 ml of 2 *M* HCl. The reduced product was extracted twice with 5 ml of ethyl acetate in the presence of 50 nmol of 3-acetyl-7-dimethylaminocoumarin as the internal standard. After centrifugation at 900 *g* for 10 min, the organic layer was evaporated *in vacuo*. The residue was reconstituted in 1.0 ml of mobile phase and subjected to HPLC. The initial reaction rate was determined from the following two factors: the decrease of NADPH absorption at 340 nm and the production of the alcohol, II, by enantiospecific HPLC. The protein concentration was determined by the method of Lowry *et al.* [17] using crystalline bovine serum albumin as the standard.

HPLC analysis

A 5-μl volume of the sample was applied to a cellulose-based chiral column, Chiralcel OD, (Daicel, Tokyo, Japan). *n*-Hexane–2-propanol (93:7, v/v) was used as the mobile phase. The isocratic chromatographic separation was performed at ambient temperature and a flow-rate of 1.2 ml/min, which resulted in an average pressure of 30 kg/cm^2. The detection wavelengths for enantiomeric alcohol and

the internal standard were adjusted to 315 nm for fluorescence excitation and 375 nm for emission.

RESULTS AND DISCUSSION

Spectral properties of I and II

It has already been demonstrated that a series of 2-phenylbenzoxazole derivatives possessed strong fluorescence, and hence some of them were applicable as sensitive fluorescence probes for trace analysis of organic compounds [18,19]. During the investigations of their fluorescence spectra, we found a remarkable difference in fluorescence properties between 4-(6-methoxy-2-benzoxazolyl)acetophenone (I) and the corresponding chemically reduced alcohol (II). The electron-donating carbinol group of II enhanced the fluorescence responses by *ca* three- to fourteen-fold above that of I, as shown in Table I. Therefore, I was suggested to be useful as a novel substrate for the sensitive assay of aromatic aldehyde or ketone reductases. However, it was necessary to improve the conventional incubation mixture owing to the extremely low solubility of I in an aqueous buffer solution (as low as 10 μM by a spectral method). The influence of the bovine serum albumin concentration on the fluorescence of I was investigated (Fig. 2). The concomitant addition of 5% acetonitrile and 1% bovine serum albumin to a reaction mixture seemed enough to achieve a complete solubilization of 500 μM substrate. Subsequent studies using rabbit liver confirmed that the enzyme activity determined by fluorescence HPLC did not exhibit any significant difference between the absence and presence of serum protein (data not shown).

TABLE I

FLUORESCENCE PROPERTIES OF 2-PHENYLBENZOXAZOLE DERIVATIVES

Compound	Solvent	Fluorescence maxima (nm)		Φ_f[a]
		Excitation	Emission	
I	CH_3CN	335	448	0 12
	C_2H_5OH	338	482	0 27
	n-Hexane	339	445	0.028
	H_2O	335	521	0.060
II	CH_3CN	315	377	0.73
	C_2H_5OH	316	377	0 75
	n-Hexane	319	357	0.38
	H_2O	312	394	0 56

[a] Fluorescence quantum yield was determined by 0.25 μM quinine sulphate in 0 05 M H_2SO_4 as the standard ($\Phi_f = 0.70$).

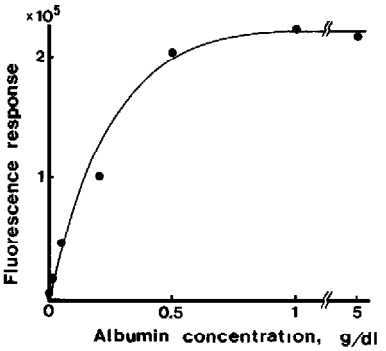

Fig 2 Effect of bovine serum albumin on the solubility of I in an aqueous buffer solution A reaction buffer (2 ml) containing 500 μM I and different concentrations of bovine serum albumin was vigorously mixed by a mechanical shaker for 10 min An aliquot of 5 μl was directly analysed using enantiospecific HPLC as described in the text The fluorescence response was expressed as the peak area calculated with a digital integration processor.

Enantiospecific HPLC separation of II in mammalian tissue preparations

A preliminary study concluded that the alcohol product, II, which was formed by enzymic reduction of I, might be directly assayed by monitoring the change of fluorescence intensity in a reaction mixture at the low concentration ranges of the substrate. However, this might cause considerable estimation errors owing to the fluorescences of substrate or cofactors, when higher concentrations are employed. Several studies of mammalian carbonyl reductase have found that most of the unsymmetrical carbonyl compound was preferentially metabolized to the corresponding *S*-alcohol as the result of enzymic reduction [12,20]. Classical spectrometry cannot be used to establish the stereochemical nature of carbonyl reductase, and several other techniques have been tried: gas chromatography [21–23], gas chromatography with mass spectrometry [24,25] and HPLC [26]. Most of these indicated the diastereomeric conversion of the assymmetric alcohol produced by reaction with an appropriate chiral reagent and subsequent separation on an achiral stationary phase, hence we had to pay attention to the reaction yield between stereoisomers and/or the racemization during derivatization [27]. Therefore, we explored the enantiomeric separation of II by means of HPLC with chiral stationary phase columns in order to achieve direct assay. Each enantiomer of II could be effectively obtained without resort to chromatography as follows: the diastereomeric conversion with (1*R*)-(−)-camphanic acid chloride to give IV, its preferential recrystallization and subsequent hydrolysis with a mild alkali.

The enantiomeric purities of both alcohol products determined by HPLC were at least 99.1%. A chiral cellulose column (Chiralcel OD) permitted the satisfactory resolution of each enantiomer without any derivatization, as shown in Table II. Typical chromatograms of extracts from rabbit liver cytosol with or without 50 nmol of racemic alcohol, II, are shown in Fig. 3A and D, respectively.

TABLE II

ENANTIOSPECIFIC HPLC SEPARATION OF II IN RABBIT LIVER CYTOSOL

Configuration of alcohol, II	t_R (min)	k'	α	R_S	Intra-assay accuracy[a] (nmol per tube)
S-(−)	12 22	5 14	1 160	1 032	9.87 ± 0 41
R-(+)	13 57	5 96			9.96 ± 0 52

[a] Five replicate tissue preparations containing 20 nmol of II (as racemate) and the internal standard were extracted and analysed by HPLC as described in text Each value is expressed mean ± S D

The retention times were 12.2 min for the *S*- and 13.6 min for the *R*-enantiomer. The substrate and the internal standard peaks were eluted at 9.5 and 19.2 min, respectively.

The calibration curve for the amount of each enantiomer *versus* the peak-area ratio to the internal standard was linear over the range 0 2–50 nmol per incubation mixture (corresponding to 1–250 pmol per injection) and passed through the origin. Intra-assay reproducibility in rabbit liver cytosol was found to be excellent, with coefficients of variation (C V.) not more than 5.2% (Table II). Amounts of 20 fmol of both IIa and IIb per injection were detectable under the conditions employed (at a signal-to-noise ratio of 3).

Biochemical properties of I for carbonyl reductase in mammalian tissues

The fluorescence HPLC assay based on the detection of the alcohol product II for the sensitive determination of carbonyl reductase activity in rabbit liver cyto-

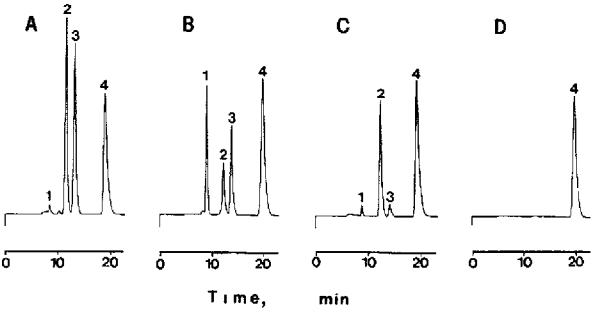

Fig 3. Enantiospecific HPLC of extracts of rabbit tissue preparations (A) A control liver cytosol containing the synthetic standards of I and II (50 nmol each). (B) Reaction mixture in the presence of 500 μM I and a kidney cytosol (C) Reaction mixture in the presence of 50 μM I and a liver cytosol (D) A control liver cytosol Peaks 1 = 4-(6-methoxy-2-benzoxazolyl)acetophenone (I), 2 = *S*-(−)-*sec*.-[4-(6-methoxy-2-benzoxazolyl)]phenethyl alcohol (IIb), 3 = *R*-(+)-*sec* -[4-(6-methoxy-2-benzoxazolyl)]phenethyl alcohol (IIa), 4 = 3-acetyl-7-(dimethylamino)coumarin (III)

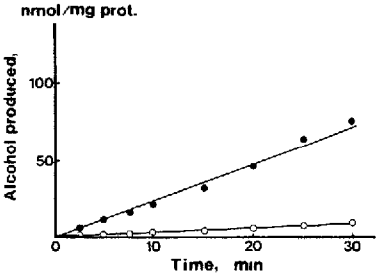

Fig. 4 Time-dependent formation of enantiomeric alcohols, *R*-(+)-isomer (IIa) (○) and *S*-(−)-isomer (IIb) (●), by enzymic reduction of I in rabbit liver cytosol.

sol was examined. The formation rates of the *S*- and *R*-enantiomers of II from 50 μM I in the presence of 200 μM NADPH were both proportional to time up to 30 min, and the former was *ca.* nine-fold higher than the latter (Fig. 4).

A typical chromatogram of an extract obtained after a 15-min reaction is shown in Fig. 3C. This finding corresponded to the current formula in the product-stereoselective metabolism of unsymmetrical carbonyl compounds, established by Baumann and Prelog [20]. NADH also catalysed the enzymic reduction of I with one third less activity than NADPH, although its stereochemical preference was so high that the *S*-isomer of II was exclusively produced (data not shown).

A rabbit liver preparation optimally reduced I at pH 6.2 (Fig. 5). The enzyme kinetic study revealed that the maximum rate of the metabolic formation of II and the apparent Michaelis constant determined for four different individuals were 2.35 ± 0.23 nmol/min/mg of protein and 9.1 ± 0.9 μM, respectively. Hence, compound I would have a higher affinity for liver cytosolic enzymes than *p*-nitroacetophenone (the apparent Michaelis constant estimated under the similar condition was 0.11 mM), which is one of the preferred substrates for the investigation of several classes of aromatic aldehyde and ketone reductases [28–30].

The present method was compared with the conventional spectrometric assay on the basis of NADPH chromogenicity (Fig. 6). The enzyme activities estimated

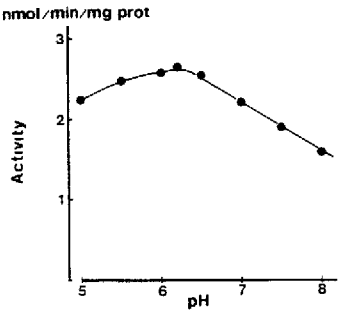

Fig. 5 pH-dependent formation of II in rabbit liver cytosol I (500 μM) reacted in 0 1 M potassium phosphate buffer containing 200 μM NADPH and was assayed as described in the text. Each point was expressed as the mean of total activity, *i e* the sum of *R*-(+)- and *S*-(−)-isomers, in three different animals.

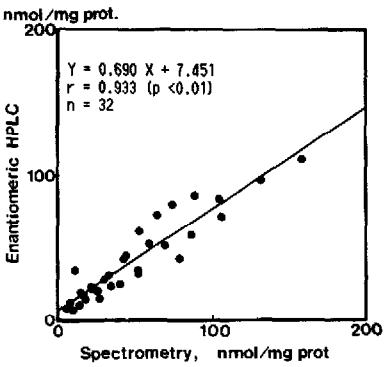

Fig 6 Comparison of assays by fluorescence HPLC and conventional spectrometry for carbonyl reductase in rabbit liver cytosol

by classical spectrometry were *ca.* 30% greater than those measured by enantiospecific HPLC, perhaps owing to a significantly biased UV absorption derived from the substrate. However, a good correlation was still observed ($r = 0.93$, $p < 0.01$).

The NADPH-catalysed metabolic reduction of I in other species or tissues was further examined, and the results are summarized in Table III. Rabbit kidney cytosol resulted in a somewhat different enantioselectivity, where more of the *R*-isomer of II was produced as shown in Fig. 3B. Thus, it was suspected that the carbonyl reductase in the rabbit kidney preparation might be highly heteroge-

TABLE III

SPECIES AND TISSUE DISTRIBUTIONS OF NADPH-DEPENDENT CARBONYL REDUCTASE ACTIVITIES AND THEIR STEREOCHEMICAL PREFERENCE

Enzyme source	Activity[a] (nmol/min/mg of protein)	Stereoselectivity[b] *S*-(−)/*R*-(+)
Rabbit		
Liver	2 15 ± 0 55	90 10
Kidney	1 12 ± 0 07	39 61
Lung	0.46 ± 0 09	91·9
Intestine	2.09 ± 0.44	93:7
Rat		
Liver	0 23 ± 0.20	83:17
Kidney	7.3 10^{-2} ± 3 5 10^{-2}	78:22
Lung	0 22 ± 0.17	73.27
Intestine	0 28 ± 0.31	78 22

[a] Mean ± S.D of four different animals

[b] Peak-area ratio of each enantiomeric alcohol produced.

neous and contain several different classes of isozymes. The *in vivo* metabolic reduction of propiophenone to its corresponding enatiomeric alcohol in rats after oral dosing resulted in greater formation of the *R*-alcohol than that observed in *in vitro* liver cytosol preparation [23]. One of the reasons for such a discrepancy may be the further preferential metabolism of alcohol, *i.e.* glucuronide conjugation. However, the present findings might also indicate that the characteristic difference in tissue distributions of isozymes may alter the stereochemical preference of the *in vivo* metabolism.

In conclusion, compound I was proved to be an excellent substrate for a highly selective and sensitive assay of mammalian carbonyl reductases. Furthermore, the enantiomeric HPLC separation of II enabled us to clarify the product-stereoselective preference of enzymes. It was also suggested that quantitative evaluation on the basis of a substrate or a product should carry more weight in research into carbonyl reductases than classical spectrometry, which measures the chromogenic change of reduced cofactors

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