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Enzymatic Characteristics of steroid Δ^1-dehydrogenase from *Arthrobacter simplex*

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Steroid Δ^1-dehydrogenase purified from hydrocortisone-induced cells of *Arthrobacter simplex* converted various 3-ketosteroids into their corresponding Δ^1-dehydrogenated products. The transformation efficiencies depend upon the chemical structure of the steroids, especially length of the side chain at 17 position and hydroxyl groups at 11 and 17 positions. The Km values for androstenedione, the most favorable substrate examined, and hydrocortisone were 74 μM and 294 μM, respectively. The optimum temperature and pH of the enzyme reaction were 35°C and pH 9, respectively, and the enzyme was relatively stable at the range from 20 to 35°C and from pH 5 to 10 after one hour of incubation. The enzyme activity was markedly inhibited in the presence of Cu^{2+}, Fe^{3+}, Hg^{2+}, Mo^{6+} ions, and somewhat inhibited by Zn^{2+} and Fe^{2+}. α, α′-Dipyridyl that inhibits 9α-hydroxylase and accumulates 1,4-androstadiene-3,17-dione from sterols revealed no inhibitory effect on this enzyme. EGTA showed inhibitory effect. β-Estradiol competitively inhibited the enzyme activity. Chemical modifications of the enzyme were attempted with several reagents. p-Hydroxymercuribenzoate showed inhibition of the enzyme activity and protection of the substrate. This suggests that cysteine residue may be involved in the active site of the enzyme.

In the field of biotechnology, steroid transforming microorganisms have been used in two areas: one is the degradation of steroid raw materials into useful intermediates for drug production and the other is the specific transformation of steroid intermediates into biologically active products. To obtain useful steroid intermediates from raw materials such as cholesterol, sitosterol, phytosterol and stigmasterol, sterol side chain cleavage should occur and nucleus degradation should be prevented. Side chain cleavage and ring degradation have been studied previously by many investigators in the studies on various microorganisms (6, 17, 18, 20-23). Basic structure of steroids is shown in Fig. 1.

Steroid Δ^1-dehydrogenation that introduces 1,2 double bond into steroid ring A is one of the critical steps of the microbiological steroid ring degradation. The microorganisms capable of dehydrogenating at the positions of C 1,2 have been used in the production of corticosteroids with increased biological activities and decreased side effects (24). For two decades the enzymatic properties of steroid Δ^1-dehydrogenase have been studied by using crude cell extracts or partially purified enzymes in several microorganisms (1, 7, 11, 15). But the purification and characterization of the enzyme was only recently accomplished with *Nocardia* (8-10). *Arthrobacter simplex* shows prominent steroid Δ^1-dehydrogenase activity, and has been used in the commercial transformation of hydrocortisone into Δ^1-dehydrogenated product, prednisolone (4, 5, 12, 13, 16, 24). Recently a steroid Δ^1-dehydrogenase of 98,000 dalton was purified from *Arthrobacter simplex* by the authors (2). It is a monomeric enzyme which dehydrogenates hydrocortisone effectively in the presence of phenazine methosulfate as an artificial electron acceptor.

In this paper the enzymatic characteristics of the steroid Δ^1-dehydrogenase from *Arthrobacter simplex* are

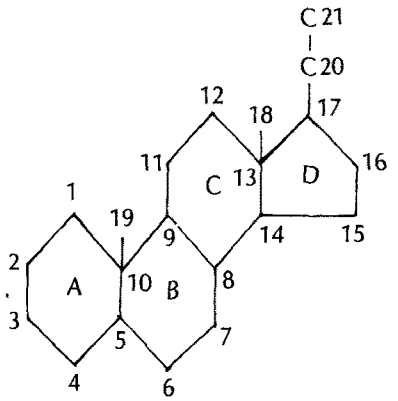

Fig. 1. Basic ring structure of the steroids.

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reported.

MATERIALS AND METHODS

Bacterial Growth and Enzyme Purification

Growth of *Arthrobacter simplex* IAM 1660 and enzyme purification from the harvested cells were accomplished by the methods presented in a previous report by the same authors (2). Enzyme used in this study was purified through streptomycin sulfate precipitation, DEAE-cellulose column chromatography and testosterone-agarose column chromatography.

Extraction and Identification of Steroids

Steroids in the whole reaction mixture were extracted three times with two volumes of ethyl acetate, and the pooled organic phases were evaporated to dryness. Residues were dissolved in ethanol and spotted to thin layer silica gel plates made of Kieselgel 60 GF_{254}. Steroids were separated with solvent system 1 (benzene : dioxane=2 : 1) or solvent system 2 (cyclohexane : ethyl acetate=4 : 6), according to their solubilities in organic solvents.

Determination of Enzyme Activity

Steroid Δ^1-dehydrogenase activity was assayed by directly measuring the amounts of reaction products on the silica gel plates using densitometer (DESAGA CD 60, Heidelberg). The enzyme reaction mixture consisted of 200 μl of 50 mM Tris buffer (pH 9.0), 20 μg of steroid substrate in 20 μl of ethanol, 20 μg of phenazine methosulfate in 20 μl of d-water, and an appropriate amount of enzyme. After the reaction at 30°C for a given period, steroids were extracted. One unit of the enzyme was defined as the amount of enzyme producing 1 μmole of prednisolone per minute.

Chemical Modification of the Enzyme

Chemical modifications of the enzyme were done by incubating the enzyme with different concentrations of the group-specific modifying reagents at a given pH and temperature for a given period (Table 1). At the end of the incubation, 200 μl of 50 mM Tris buffer (pH 9.0), 10 μg of 4-androstene-3,17-dione and 10 μg of phenazine methosulfate were added to the modification reaction mixtures, and the enzyme reaction mixture were incubated at 30°C for another given period. The enzyme activities were described as the relative activity when the transformation efficiency of the reaction without modification reagents was considered as 100%. Protection of the reactive groups by substrate against the modifiers was accomplished by adding the substrate before the modification reactions. Modifications with N-acetylimidazole, p-hydroxymercuribenzoate, diethylpyrocarbonate and N-bromosuccinimide were done according to Boopathy and Balasubramanian (3).

Table 1. Experimental conditions for chemical modification of steroid Δ^1-dehydrogenase

Modifying reagents	Conditions of reaction
N-Bromosuccinimide (NBS)	in 10 mM citrate (pH 5.0), at 37°C for 15 min
Diethylpyrocarbonate (DEP)	in 10 mM Tris (pH 7.0), at 22°C for 15 min
N-Acetylimidazole (NAI)	in 10 mM Tris (pH 7.0), at 37°C for 20 min
p-Hydroxymercuribenzate (p-HMB)	in 10 mM Tris (pH 8.0), at 37°C for 30 min
Iodoacetamide (IAM)	in 10 mM Tris (pH 7.0), at 37°C for 60 min
Pyridoxal-5-phosphate (PLP)	in 10 mM Tris (pH 7.0), at 37°C for 30 min

RESULTS AND DISCUSSION

Substrate Specificity of the Steroid Δ^1-Dehydrogenase from *Arthrobacter simplex*

Substrate specificity of the enzyme was examined by the standard assay method, however hydrocortisone was substituted with other various steroids. Δ^1-Dehydrogenated products were identified by comparing their Rf values and colors with the authentic steroids on the thin layer chromatograms after the treatment with sulfuric acid spray followed by heating at 150° for a few minutes. Steroid Δ^1-dehydrogenase from *Arthrobacter simplex* revealed a wide substrate spectrum of 3-ketosteroids. Various 3-keto-4-ene steroids were converted to the corresponding 1-dehydrogenated products (Fig. 2). Transformation efficiency of each steroid was calculated from the amount of the product on densitogram (Table 2). Although the enzyme was induced with hydrocortisone, purified enzyme did not dehydrogenate hydrocortisone at the highest efficiency. 4-Androstene-3,17-dione was the best substrate and progesterone showed similar result. Relative transformation ratio describes the ratio of transformation efficiency of each substrate to that of 4-androstene-3,17-dione, the most favorable substrate. Transformation efficiencies depend on the chemical structures of steroids. Cholestenone with long side chain at 17 position was converted at low efficiency, possibly due to steric hindrance. Hydroxyl groups at 11α, 11β and 17 position and 17-ethynyl group also revealed hindering effects. Although 11-hydroxyl group showed inhibitory effect, the enzyme still dehydrogenated steroids with 11-hydroxyl groups at considerable rates compared to the enzyme from *Nocardia* that dehydrogenated hydrocortisone or 11-hydroxy-

progesterone at lower efficiency (8). Crude enzyme from *Pseudomonas* revealed no activity with 11-keto or 11-hydroxy steroids (14). Δ^1-Dehydrogenase from *Arthrobacter simplex* is important due to its substrate specificity in which hydrocortisone can be converted to prednisolone at higher transformation efficiency than the enzymes of other microorganisms. Norethindrone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one) was converted to its estradiol form, 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol (Fig. 2).

Effects of Temperature

Enzyme reactions were performed at various temperatures of 20, 25, 30, 35, 40, 45, 50 and 55°C, respectively. The optimum reaction temperature was 35°C (Fig. 3). Temperature stability of this enzyme was tested. After 1 hour of incubation at various temperatures of 20, 25, 30, 35, 40, 45, 50 and 55°C, enzyme reactions were performed at 30°C. The enzyme was relatively stable at 20~35°C, and marked decrease in activity was observed above 40°C. At 45°C nearly 50% of activity was lost (Fig. 3).

Effects of pH

Enzyme reactions were performed at various pHs of 3, 4, 5, 6, 7, 8, 9, 10 and 11, respectively. The optimum pH for reaction was 9 (Fig. 4), which is close to that of *Nocardia* of pH 10 (8) and *Pseudomonas* of pH 9 (15). Δ^1-Dehydrogenase from *Arthrobacter simplex* has a wider pH range compared to that of *Pseudomonas*

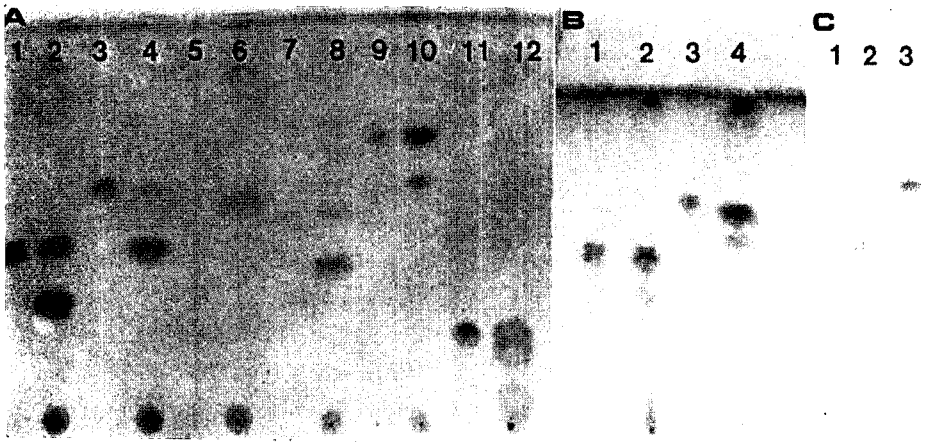

Fig. 2. Thin layer chromatograms of various steroid substrates and Δ^1-dehydrogenated products.
Spots in the lane of the odd numbers are substrate steroids and spots in the lane of the even numbers are extracted substrates and products after the enzyme reactions as described in methods. A: 1. Testosterone (TS), 2. TS, Δ^1-TS, 3. Androstenedione (AD), 4, AD, Δ^1-AD (ADD), 5. Progesterone (PG), 6. PG, Δ^1-PG, 7. 17-OH-PG, 8. 17-OH-PG, Δ^1-17-OH PG, 9. Ethisterone (ETH), 10. ETH, Δ^1-ETH, 11. Hydrocortisone acetate (HCAc), 12. HCAc, Δ^1-HCAc. B: 1. Hydrocortisone (HC), 2. HC, Δ^1-HC (Prednisolone, PDL), 3. 11-OH-PG, 4. 11-OH-PG, Δ^1-11-OH-PG. C: 1. Norethindrone (NEN) 2. NEN, Ethynylestradiol, 3. Ethynyl estradiol.

Table 2. Substrate specificity of steroid Δ^1-dehydrogenase from *Arthrobacter simplex*

Substrate	Transformation efficiency* (%)	Relative transformation ratio
4-Androstene-3,17-dione	79.4	1.00
Progesterone (4-Pregnene-3,20-dione)	74.6	0.94
Testosterone (4-Androstene-17β-ol-3-one)	68.5	0.86
17α-Hydroxyprogesterone	41.7	0.53
Hydrocortisone acetate	35.2	0.44
11α-Hydroxyprogesterone	28.6	0.36
Hydrocortisone (4-pregnene-11β,17α,21-triol-3,20-dione)	26.7	0.34
Ethisterone (4-androstene-17α-ethynyl-17β-ol-3-one)	20.1	0.25
Cholestenone (4-cholestene-3-one)	0.8	0.01
Norethindrone (4-pregnene-19-nor-17-ol-20-yn-3-one)	nd**	
Pregnenolone (5-Pregnene-3-ol-20-one)	0.0	0.00
Cholesterol (5-Cholestene-3-ol)	0.0	0.00

* Transformation efficiency represented the relative amount of the Δ^1-dehydrogenated product from each substrate when the amount of substrate was 100 and relative transformation ratio was the ratio of transformation efficiency of each substrate to that of 4-androstene-3,17-dione, the most favorable substrate. ** Not determined due to the conversion of the substrate to ethynyl estradiol (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol).

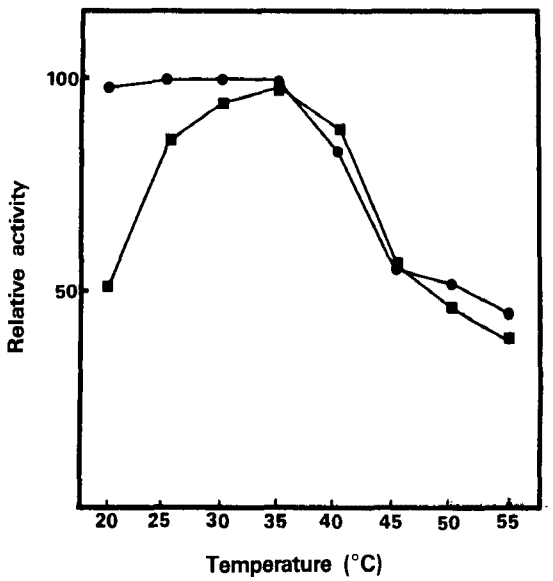

Fig. 3. Optimum reaction temperature (■) and thermal stability (●) of the steroid Δ^1-dehydrogenase of *Arthrobacter simplex*.
Effects of temperatures on the activity of the enzyme were examined by performing the enzyme reactions at various temperatures in 50 mM Tris-HCl buffer (pH 9.0) containing 20 μg of hydrocortisone and 20 μg of phenazine methosulfate in the total volume of 260 μl. Thermal stability of the enzyme was tested by assaying the activity at 30°C after one hour of incubation at various temperatures.

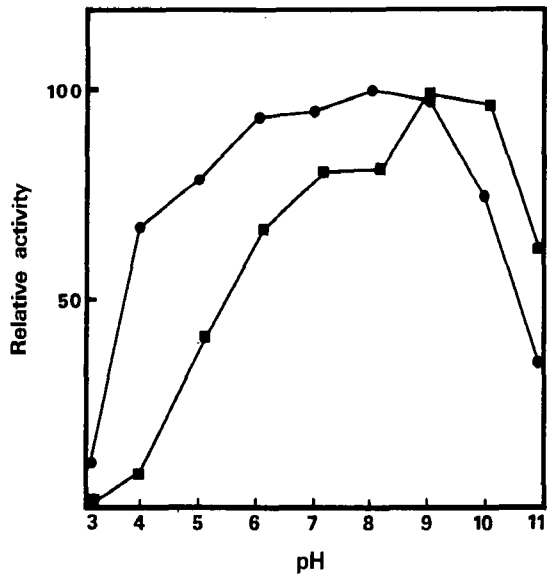

Fig. 4. Optimum reaction pH (■) and pH stability (●) of the steroid Δ^1-dehydrogenase.
Effects of pH on the activity of the enzyme were examined by performing the enzyme reactions at various pHs using three kinds of buffer; 10 mM citrate buffer for pH 3~6, 10 mM Tris buffer for pH 7~9 and 10 mM CAPS buffer for pH 10~11. The pH stability of the enzyme was tested by assaying the activity at pH 9 after 1 hour of incubation at various pHs.

in which no activity was detected below pH 7.4 (14). Stability of the enzyme at various pHs was tested. After 1 hour of the incubation at pH 3, 4, 5, 6, 7, 8, 9, 10 and 11, enzyme activities were assayed at pH 9. The enzyme was relatively stable within a pH range from 5 to 10,

Table 3. Effects of metal ions on the enzyme activity of steroid Δ^1-dehydrogenase using hydrocortisone as a substrate

Matal ions	Relative activity (%)*	
	1 mM	10 mM
None	100	100
Na^+	97	97
Ag^+	86	87
K^+	104	104
Li^+	100	100
Zn^{2+}	93	69
Cu^{2+}	84	34
Co^{2+}	90	78
Ba^{2+}	83	79
Ca^{2+}	100	100
Mn^{2+}	94	100
Hg^{2+}	83	42
Fe^{2+}	100	65
Pb^{2+}	100	86
Mg^{2+}	100	106
Fe^{3+}	100	0
Mo^{6+}	100	38

* Enzyme activity without addition of metal ion was represented as 100.

Table 4. Effects of chelating agents on the enzyme activity of steroid Δ^1-dehydrogenase

Ion chelators (10 mM)	Relative activity (%)*
None	100
α,α'-Dipyridyl	92
o-Phenanthroline	92
EDTA	96
EGTA	20

* Enzyme activity without addition of chelating agent was represented as 100.

and more stable at alkaline pH (Fig. 4).

Effects of Various Compounds on the Enzyme Activity

The effects of various metal ions on this enzyme activity were examined and the results were listed in Table 3. The enzyme was markedly inhibited by Cu^{2+}, Fe^{3+}, Hg^{2+} and Mo^{6+} and somewhat inhibited by Zn^{2+} and Fe^{2+}. These results are similar to that of *Pseudomonas* (15) except that Mn^{2+} had no inhibitory effect on the enzyme of *Arthrobacter simplex*.

Several ion chelators were tested for their effects on the enzyme activity (Table 4). EGTA showed strong inhibitory effect, suggesting Ca^{2+} ion might be involved in the enzyme activity.

Effects of the substrate analogs were tested (Table 5) and estrogens showed inhibition of enzyme activity. The extent of the inhibition varied according to their chemical structures.

Kinetics of the Steroid Δ^1-Dehydrogenase

Table 5. Effects of substrate analogs on the enzyme activity of steroid Δ^1-dehydrogenase

Steroids (160 μg/ml)	Relative activity (%)*
None	100
Estriol	62
17α-Estradiol	93
17β-Estradiol	80
17α-Ethynyl estradiol	41

* Enzyme activity without substrate analog was represented as 100.

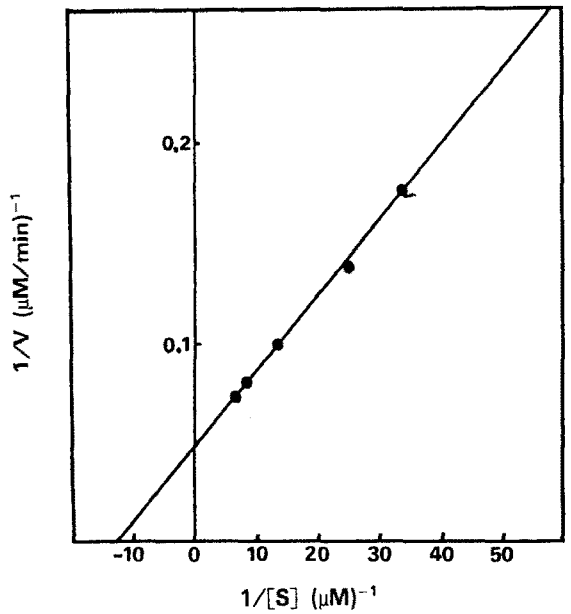

Fig. 5. Lineweaver-Burk plot of steroid Δ^1-dehydrogenase using 4-androstene-3,17-dione as a substrate.
The Km value for 4-androstenedione was 74 μM.

Double reciprocal plots of enzyme activity versus substrate concentrations showed Km values of 74 μM for 4-androstene-3,17-dione (Fig. 5), and 294 μM for hydrocortisone (Fig. 6). Double reciprocal plots of enzyme activity versus 4-androstene-3,17-dione concentrations in the presence of β-estradiol at the concentrations of 117 μM and 176 μM showed competitive inhibition of β-estradiol (Fig. 7).

Effects of Chemical Modifying Reagents

Several kinds of chemical modifying reagents were tested for their effects on the enzyme activity (Table 6). Modification reactions were performed both in the absence and in the presence of the substrate, so that the occurrence of the substrate protection could be tested. In the reagents tested, p-hydroxymercuribenzoate showed markedly decreasing effects on the enzyme activity and substrate protection. Iodoacetamide also showed inhibition and substrate protection, but by less extent. These results suggest that -SH group(s) plays an important role in the catalyzing reaction, and cysteine residue(s) may be involved in the active site

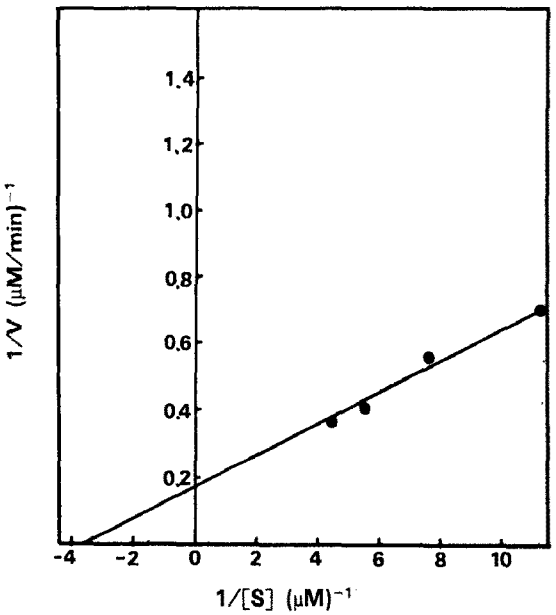

Fig. 6. Lineweaver-Burk plot of steroid Δ^1-dehydrogenase using hydrocortisone as a substrate.
The Km value for hydrocortisone was 294 μM.

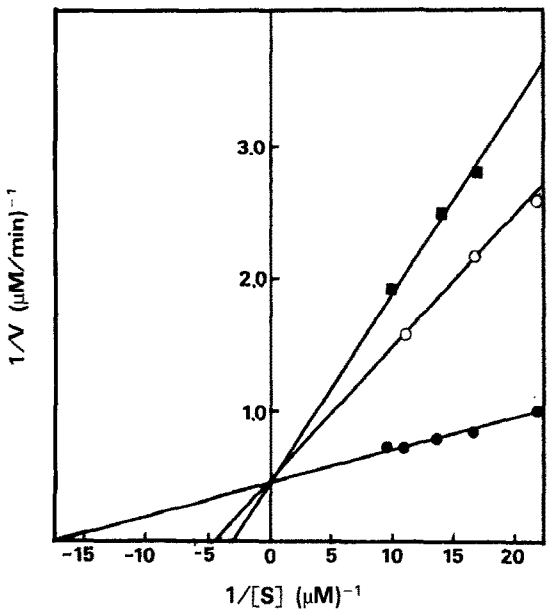

Fig. 7. Competitive inhibition of β-estradiol at two differentconcentrations for the activity of steroid Δ^1-dehydrogenase when 4-androstene-3,17-dione was used as a substrate.
No inhibitor was added (●), 117 μM of β-estradiol was added(○)and 176 μM of β-estradiol was added (■), respectively.

of the enzyme. Similar results were reported with partially purified enzyme in *P. testosteroni* (15).

Δ^1-Dehydrogenase purified from *Arthrobacter simplex* shows some differences compared to that of other microorganisms. Molecular weight (98,000) is much larger than those of *Pseudomonas testosteroni* of 59,000 (19) and *Nocardia corallina* of 60,500 (8). Substrate specificity is also different. Somewhat high conversion efficiency of hydrocortisone having three hydroxyl groups which

Table 6. Effects of group-specific modifying reagents on the activity of steroid Δ^1-dehydrogenase

Affected amino acid	Chemical modifying reagents	Conc. (mM)	Relative activity* (%)	Substrate protection** (%)
Cys	p-Hydroxymercuribenzoate (p-HMB)	0	100	
		0.2	29	78
		1.0	8	72
	Iodoacetamide (IAM)	0	100	
		1.0	81	99
		5.0	78	99
Trp	N-Bromosuccinimide (NBS)	0	100	
		0.01	70	–
		0.05	80	–
Tyr	N-Acetylimidazole (NAI)	0	100	
		10	25	–
		100	26	–
Lys	Pyridoxal-5-phosphate (PLP)	0	100	
		0.1	92	–
		1.0	70	–
His	Diethylpyrocarbonate (DEP)	0	100	
		0.5	99	
		2.0	50	–

* 4-Androstene-3,17-dione was used as substrate and the enzyme activity without modifying reagent was represented as 100.
** Substrate protection was tested by the addition of 4-androstene-3,17-dione in the modifying reaction mixture.

reveal steric hindrance could explain why *Arthrobacter simplex* has been used for the production of prednisolone in the steroid drug industry.

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