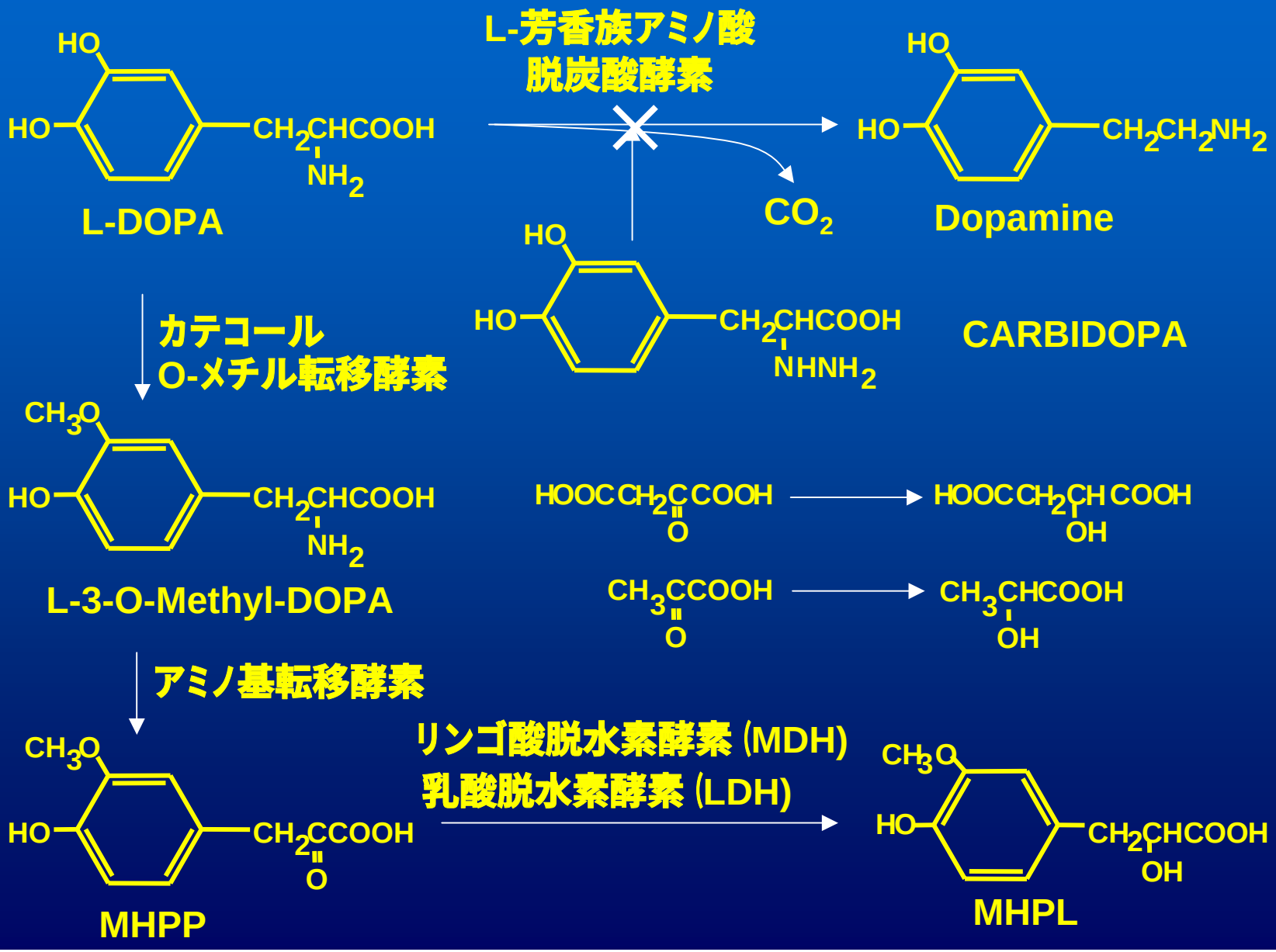
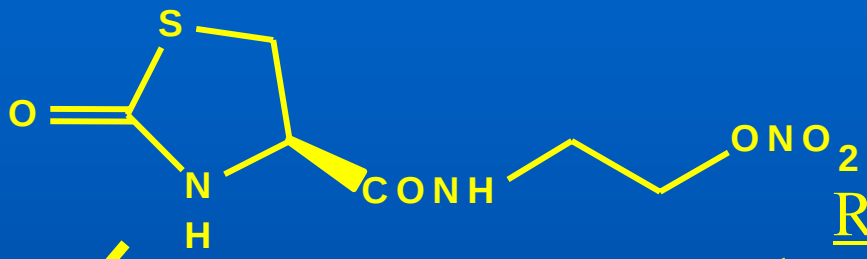
新規な薬物代謝反応および代謝酵素機能に関する研究

三共株式会社薬剤動態研究所
池田敏彦

酵素の基質特異性の厳密性?



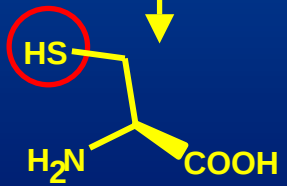
RS-7897
A New Vasodilating Organic Nitrate



RS-7897

What amidase?

L-2-Oxothiazolidine-4-carboxylic acid (L-OTCA)



L-Cysteine

Aminoethylnitrate (AEN)

Antagonize “Nitrate tolerance”

Potent vasodilating activity

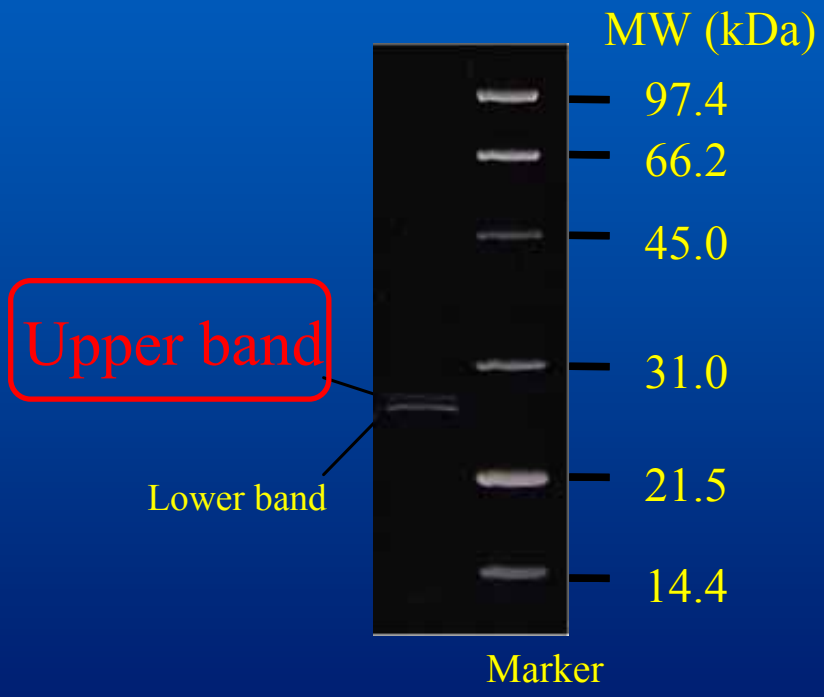
Purification of RS-7897 Hydrolase from Rat Liver

Step	Total activity (nmol/min)	Specific activity (nmol/min/mg- protein)	Purification factor	Recovery (%)
Homogenate	1012	0.04	1.0	100.0
Cytosol	693	0.11	2.7	68.5
$(NH_4)_2SO_4$ ppt.	576	0.30	7.5	56.9
DEAE Sephacel	163	0.60	15.0	16.1
Superdex™ 75	145	17.44	439.1	14.3
1st Hydroxylapatite	70	49.57	1248.3	6.9
2nd Hydroxylapatite	10	131.83	3319.9	1.0

Identification of RS7897 Hydrolase as PAP-I (Pyroglutamyl Aminopeptidase I)

SANKYO

SDS-PAGE of purified fraction



In-gel trypsin digestion

↓

nano-LC/ESI-MS/MS analysis

↓

NCBI nonredundant database

⇩

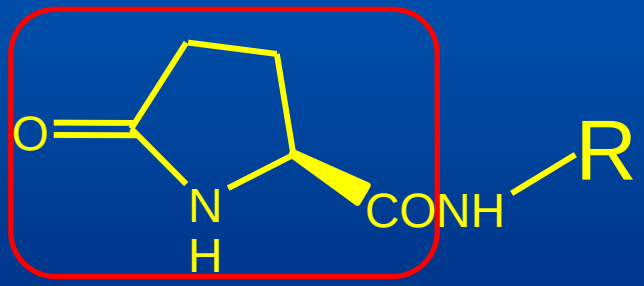
putative mouse Pyroglutamyl Aminopeptidase I (PAP-I)

(gi 12963583)

Pyroglutamyl Aminopeptidase I (PAP-I)

L-OTCA moiety

RS-7897



L-Pyroglutamyl moiety

PAP-I

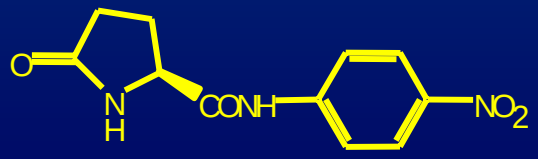
L-Pyroglutamic acid (L-pGlu)

+ NH_2-R

L-pGlu-His-Pro-NH_2 TRH

L-pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 LHRH

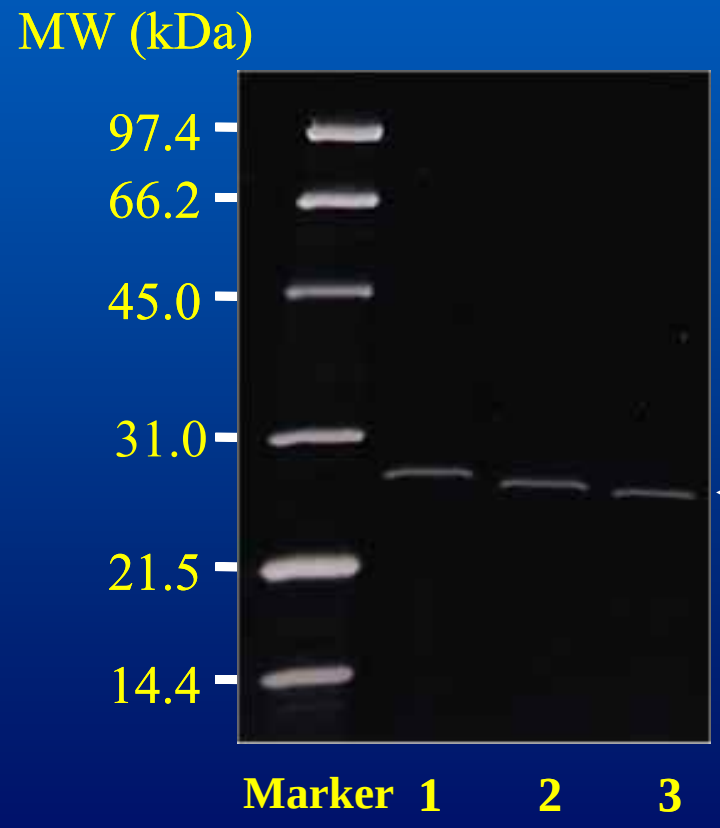
L-pGlu *p*-nitroanilide



L-pGlu-*p*NA

Expression and Purification of Recombinant PAP-Is

Rat, mouse and human PAP-Is were expressed by transfecting *E. coli* with cDNA.



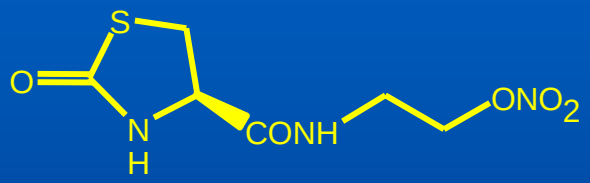
PAP-I 23-25 kDa

Lane 1：rat PAP-I
Lane 2：mouse PAP-I
Lane 3：human PAP-I

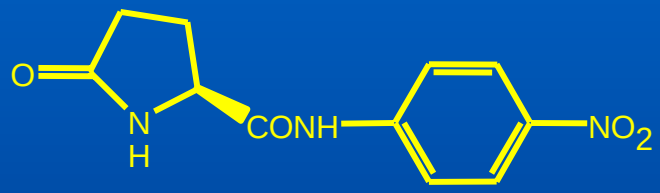
Comparison of Activities of Different PAP-Is

Species	Substrates: RS-7897 K_m (mM)	V_{max} (μmol/min/mgP)	V_{max}/K_m (ml/min/mgP)	Substrates: L-pGlu-*p* NA K_m (mM)	V_{max} (μmol/min/mgP)	V_{max}/K_m (ml/min/mgP)
Purified PAP-I						
Rat liver	0.41	3.4	8.4	0.030	5.4	176.0
Recombinant PAP-I						
Rat	0.55	6.0	10.8	0.054	17.7	327.7
Mouse	0.58	5.8	10.0	0.038	8.0	209.9
Human	0.50	4.7	9.4	0.038	10.8	280.9

RS-7897



L-pGlu-*p* NA

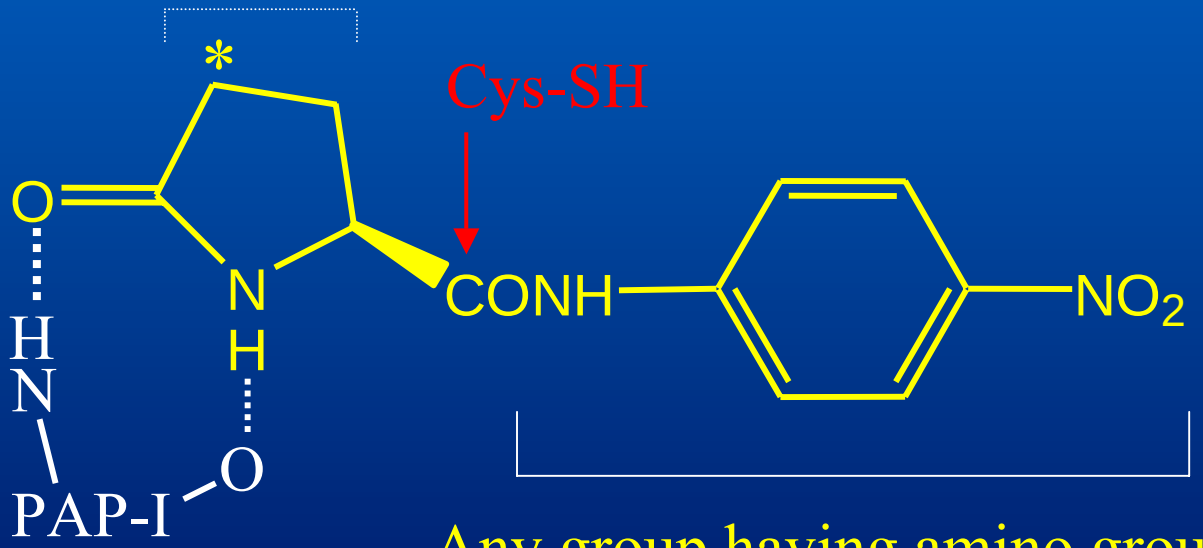


mgP: mg protein

Substrate Recognition by PAP-I

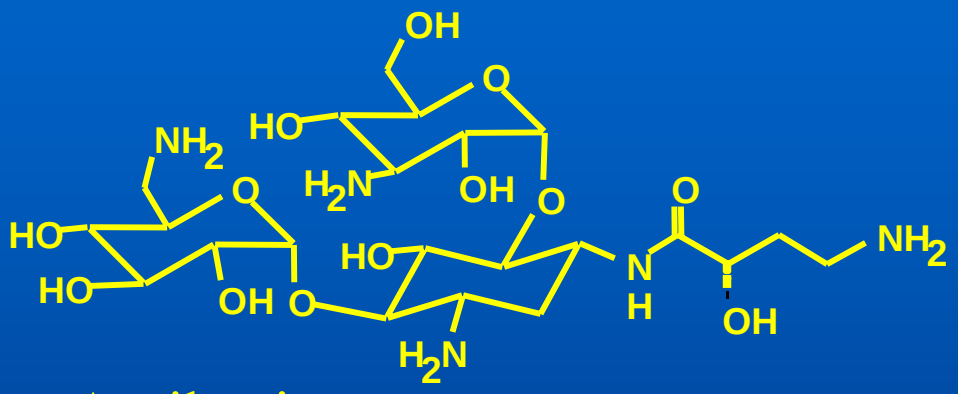
Hydrophobic pocket of PAP-I

*: C > S, O, N as substrate



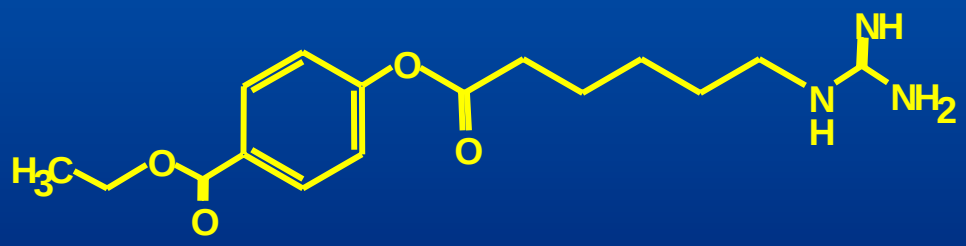
Any group having amino group is acceptable as substrate except for proline.

Possibility of Prodrug Conversion of Amino-Compounds



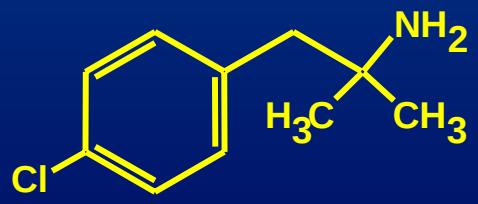
Amikacin

Arbecacin
Butirosin
Capreomycin
Destomycin A
Dibekacin
Enviomycin
Fortimicin
Gentamicin
Isepamicin
Kanamycin
Neomycin
Netilmicin
Paromomycin
Ribostamycin
Sisomicin
Tuberactinomycin



Gabexate

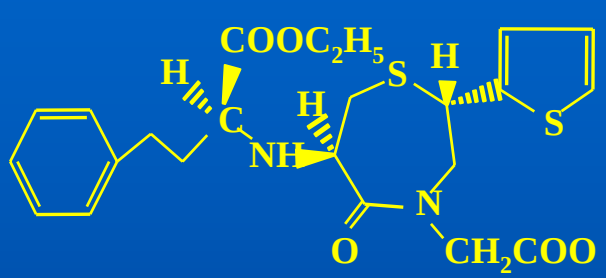
Camostat
Butirosin
Lamifiban
Melagatran
Nafamostat
Xemilofiban



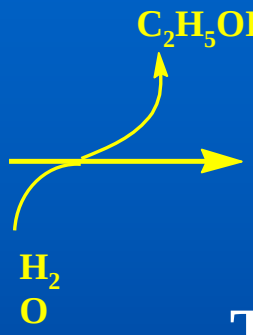
Chlorphentermine

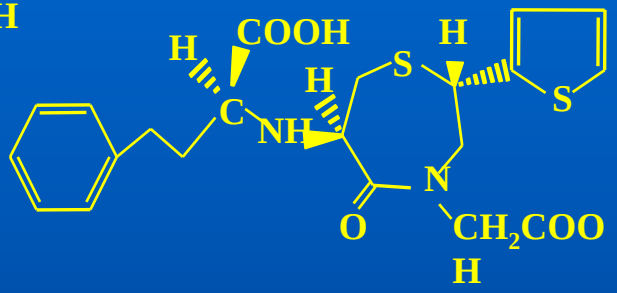
Clortermine
Butirosin
Mexiletine
Midodrine
Primaquine

Two Prodrugs Related to Angiotensin II (AII)

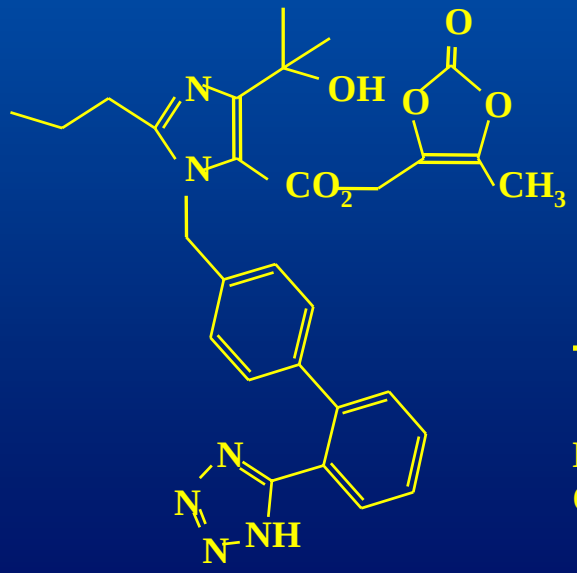


Temocapril

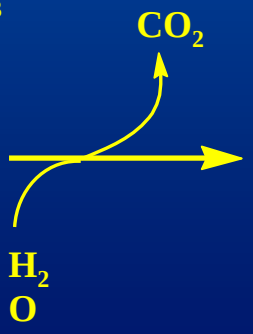


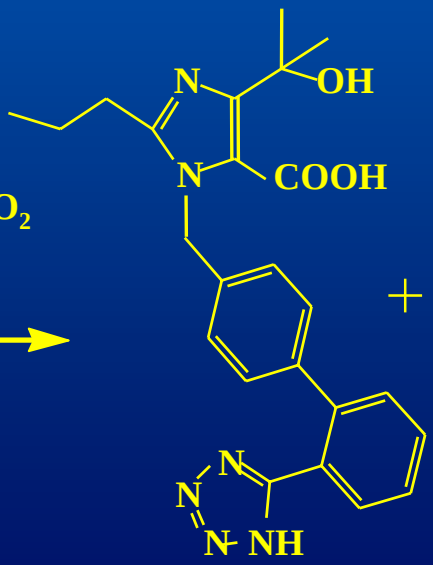


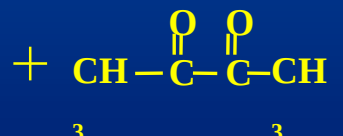
Temocaprilat (ACE inhibitor)



Olmesartan medoxomil



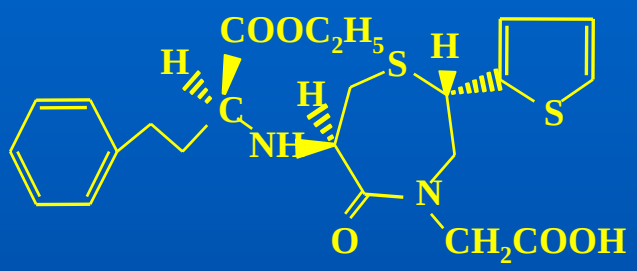




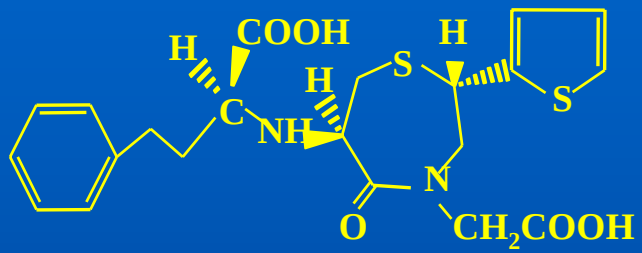
Diacetyl

Olmesartan (AII blocker)

Metabolic Pathway of Temocapril

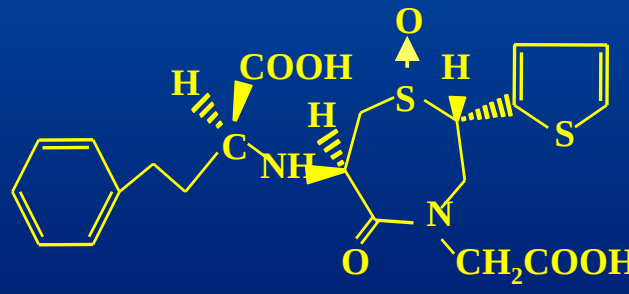


Temocapril



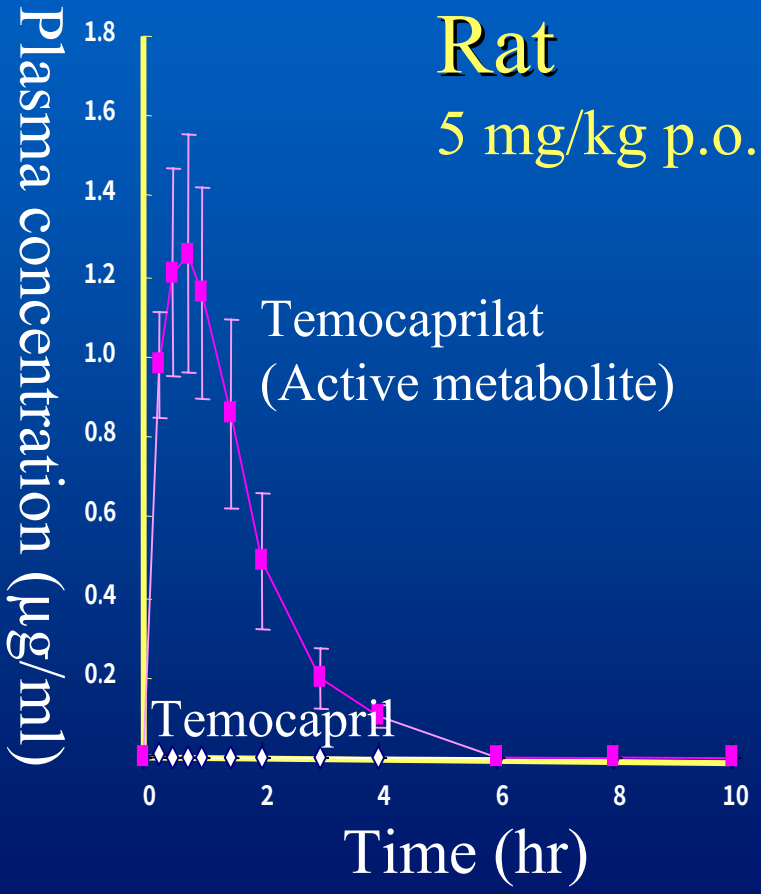
Temocaprilat

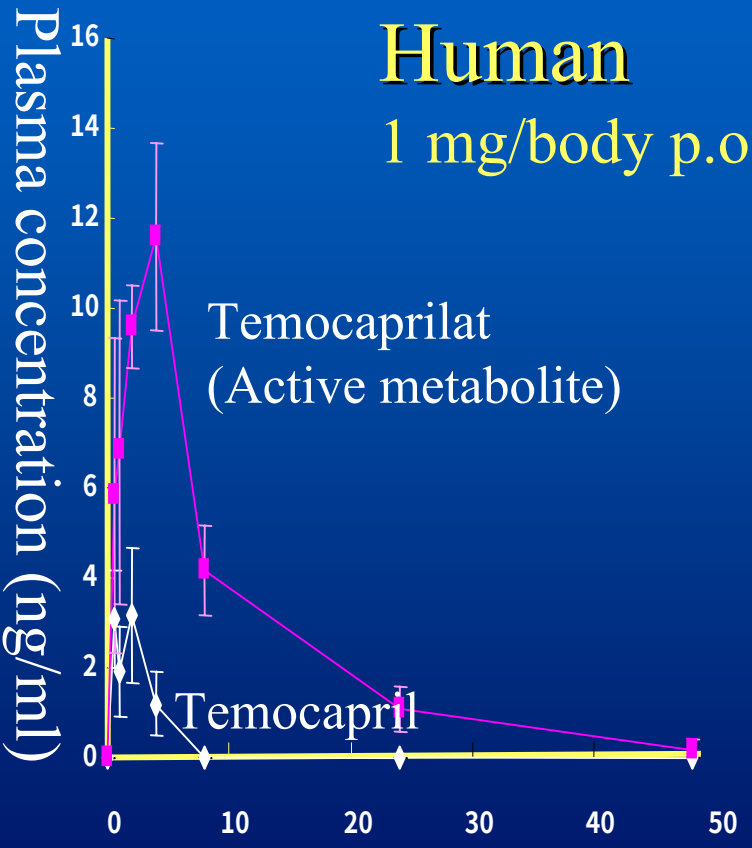
Bile & Urine



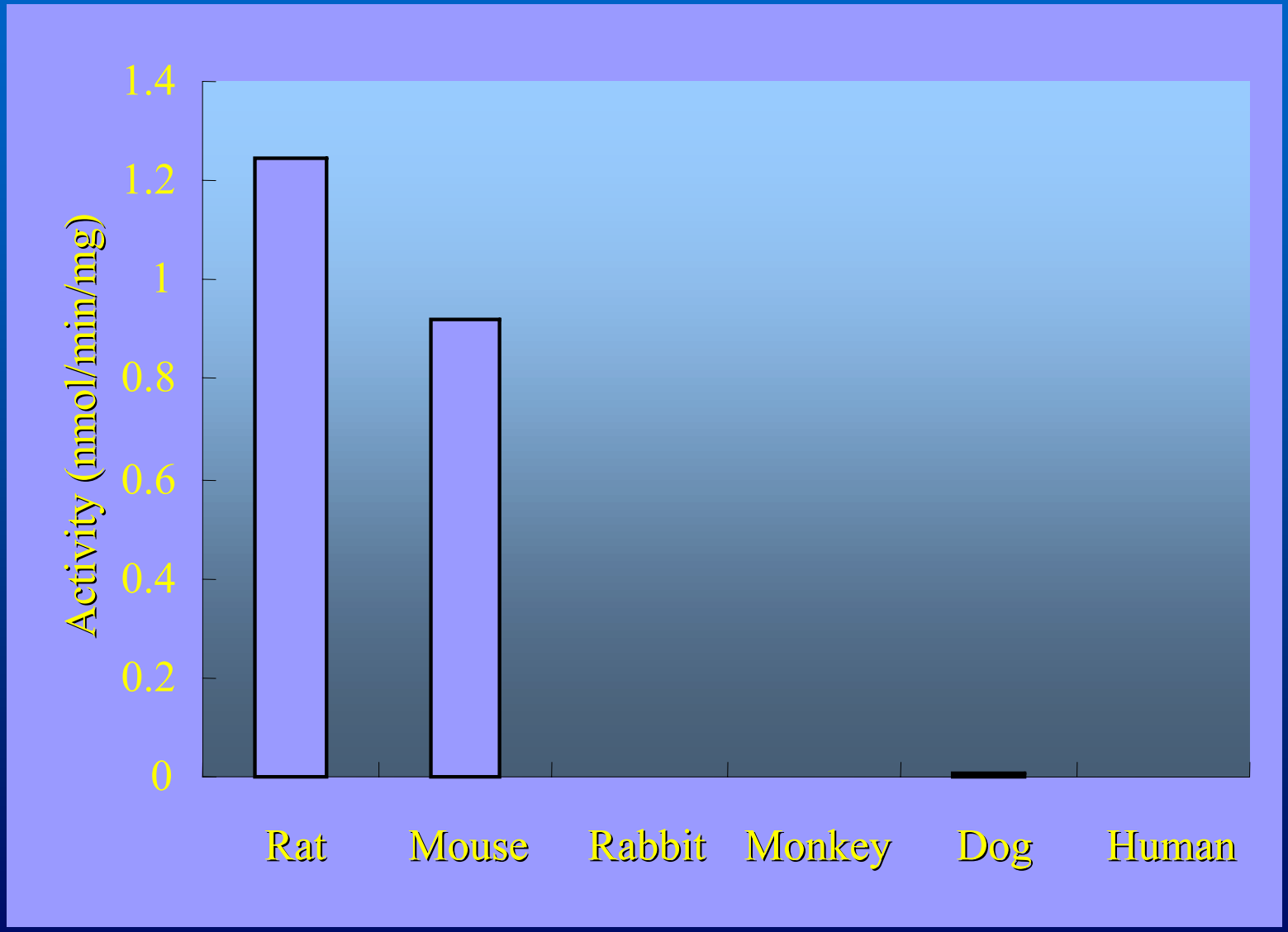
Temocaprilat S-oxide

Plasma Concentrations after Oral Administration of Temocapril to Rat and Human





Hydrolysis of Temocapril in Plasma



Purification of Temocapril-Esterase from Rat Plasma

Purification step \ Substrate	Temocapril		α-Naphthyl acetate		Benzoyl choline	
	Activity[1)]		Activity[2)]		Activity[3)]	
Plasma	1.4	(1)	0.19	(1)	0.54	(1)
$(NH_4)_2SO_4$	1.2	(0.9)	0.17	(0.9)	0.55	(1)
DEAE-Sephacel (1)	2.1	(1.5)	0.32	(1.7)	0.65	(1.7)
DEAE-Sephacel (2)	4.6	(3.4)	0.63	(3.3)	0.94	(2.6)
DEAE-HPLC	7.5	(5.5)	1.20	(6.3)	1.42	(0.04)
Hydroxylapatite	50.7	(37.3)	6.94	(36.3)	0.02	(0.0)
Hydrophobic Inter.	90.9	(66.9)	9.97	(52.2)	0.0	(0.0)
Gel permeation	293.0	(215.6)	26.8	(140.4)		
TEAE-HPLE	281.9	(207.5)	12.0	(62.7)		

1)nmol/min/mg-protein 2) μmol/min/mg-protein 3)nmol/min/mg-protein

N-Terminal Amino Acid Sequence of Temocapril-Esterase

H_2N-Gly -Pro-Ser-Ser-Pro- X -Val-Val-Val-Thr-Thr-

CES1A family

Rat liver carboxylesterases[1)]

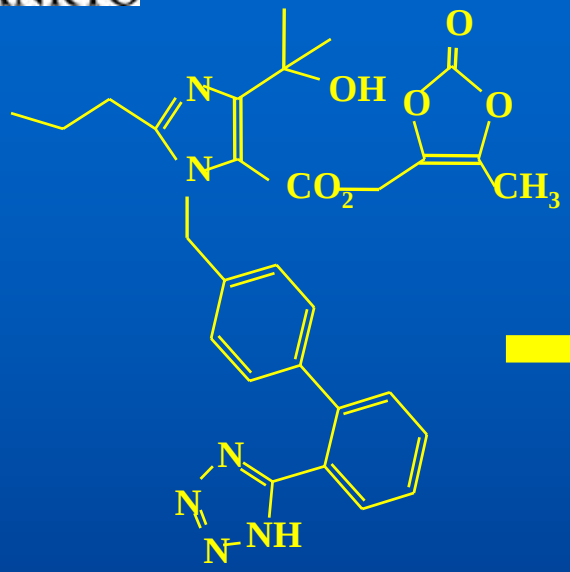
RL1: H_2N-Asp-Pro-Ser- X -Pro-Pro-Val-Val-Asp-Thr-Val-
RL2: H_2N- X -Pro-Ser- X -Pro-Pro-Val-Val-Asn- X -Val-
RH1: H_2N-Tyr-Pro-Ser- X -Pro-Pro-Val-Val-Asn- X -Val-

Human liver carboxylesterase[1)]

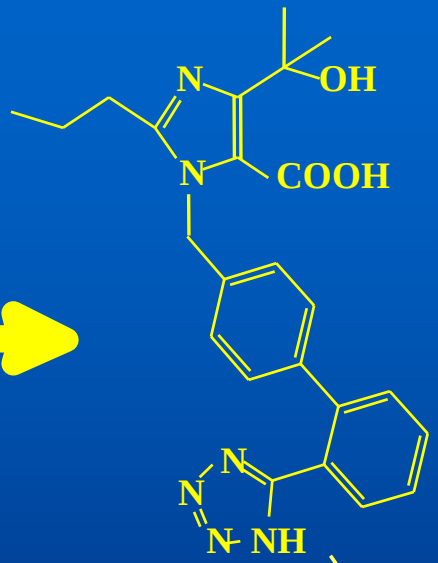
HU1: H_2N-Gly-Pro-Pro-Ser-Pro-Pro-Val-Val-Asp-Asp-Val-

1) M.Hosokawa, *Xenobio. Metabol. Dispos*. **5**, 953 (1990)

Metabolic Pathway of Olmesartan Medoxomil



Olmesartan Medoxomil



Olmesartan

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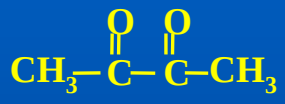
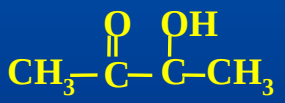


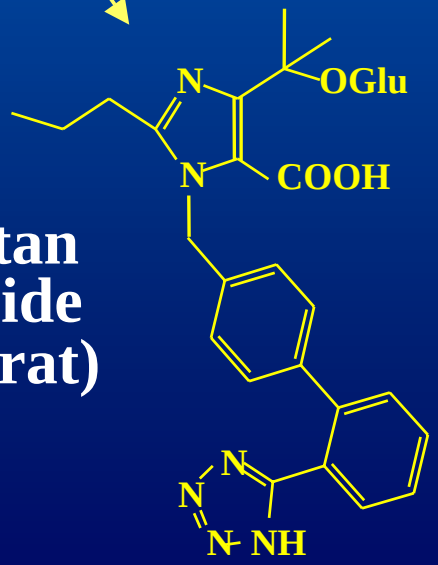
Diacetyl



Acetoin

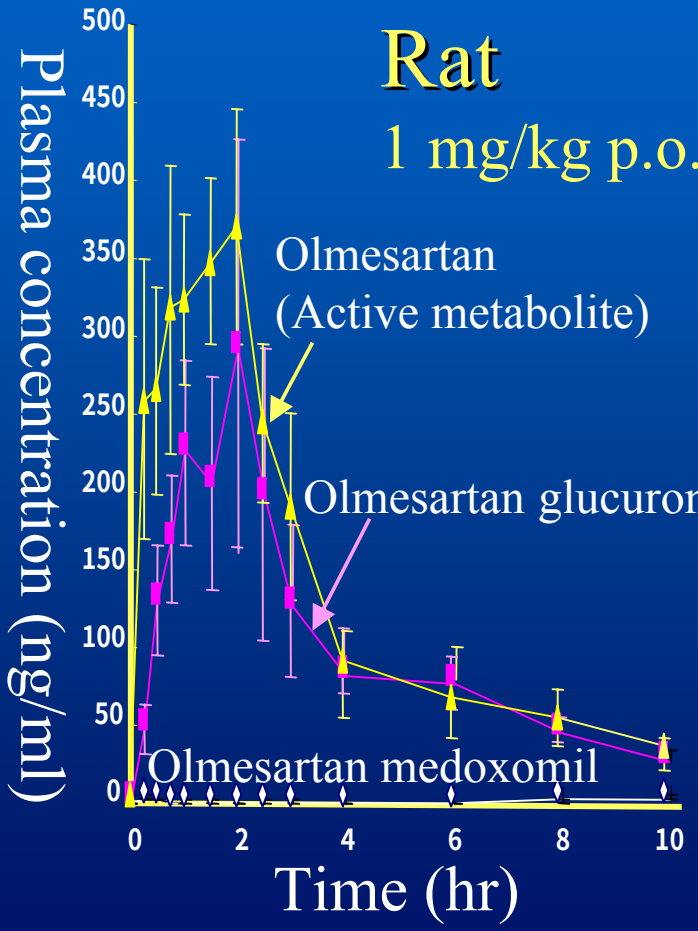
Butanediol

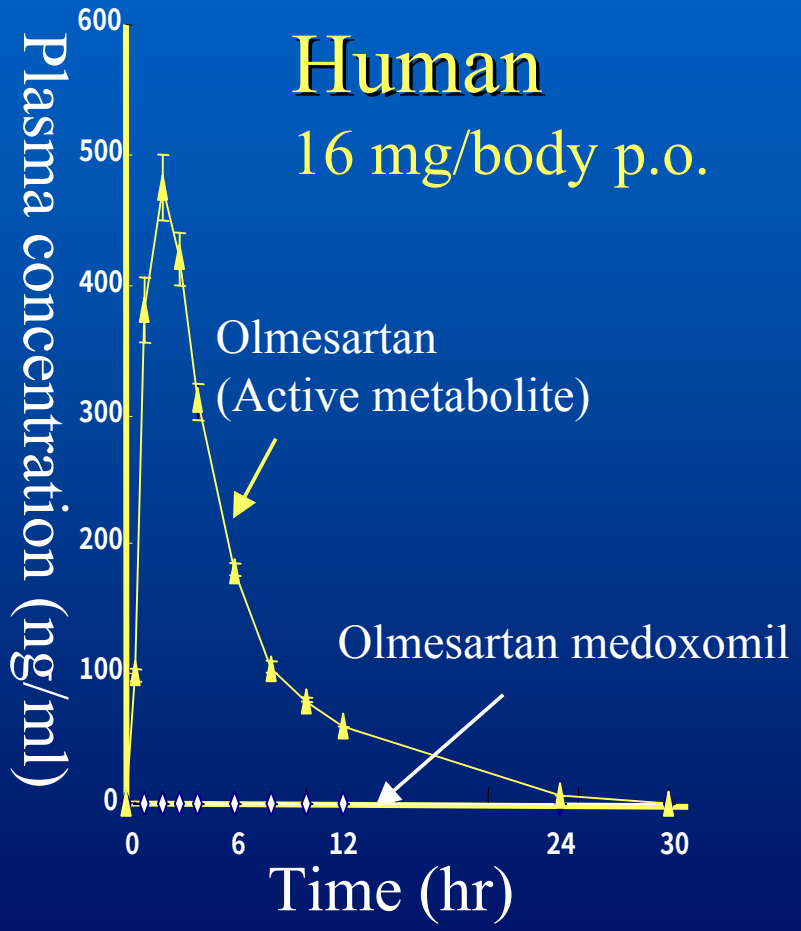
Bile & Urine



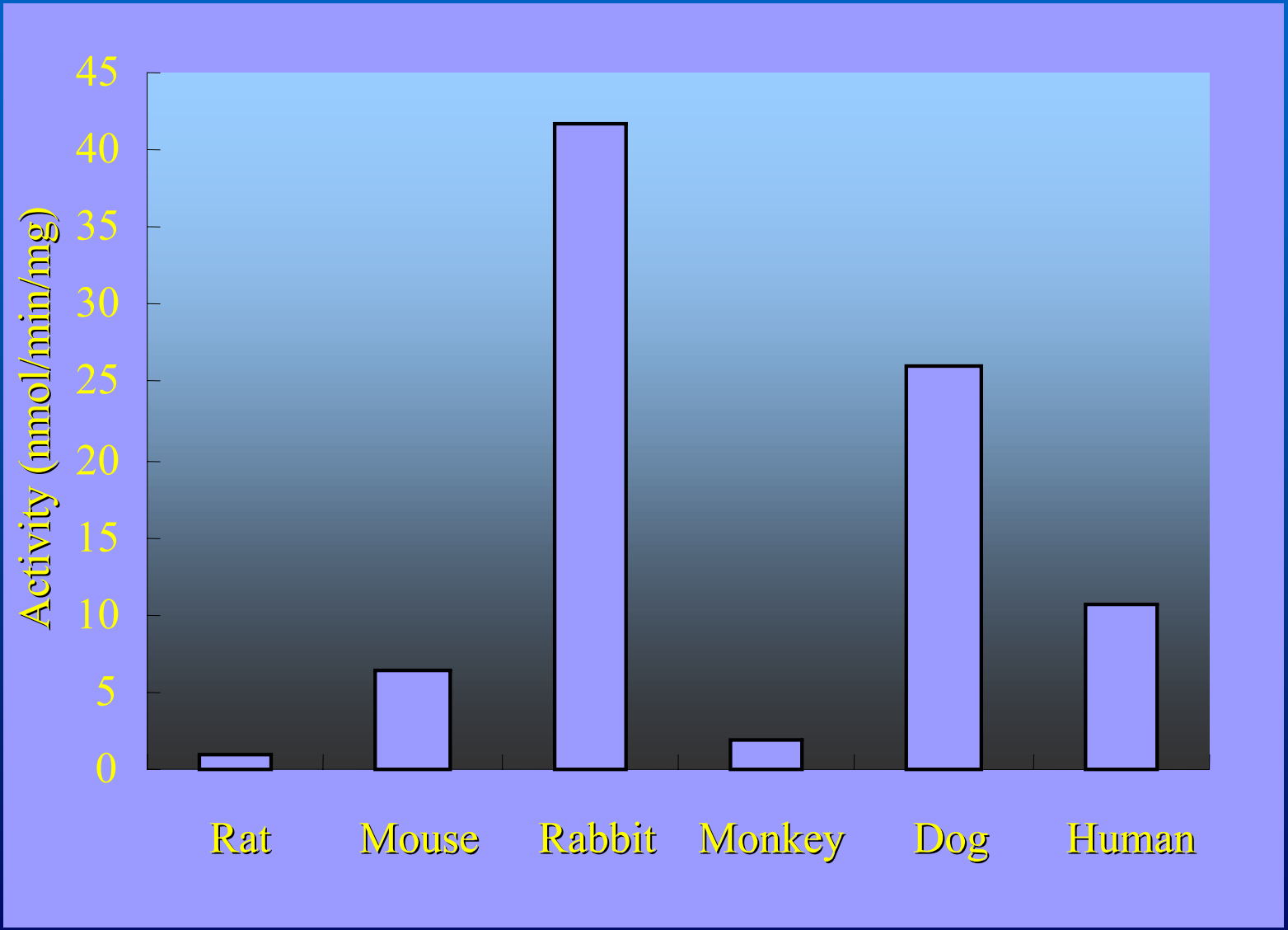
Olmesartan glucuronide (Only in rat)

Plasma Concentrations after Oral Administration of Olmesartan Medoxomil to Rat and Human

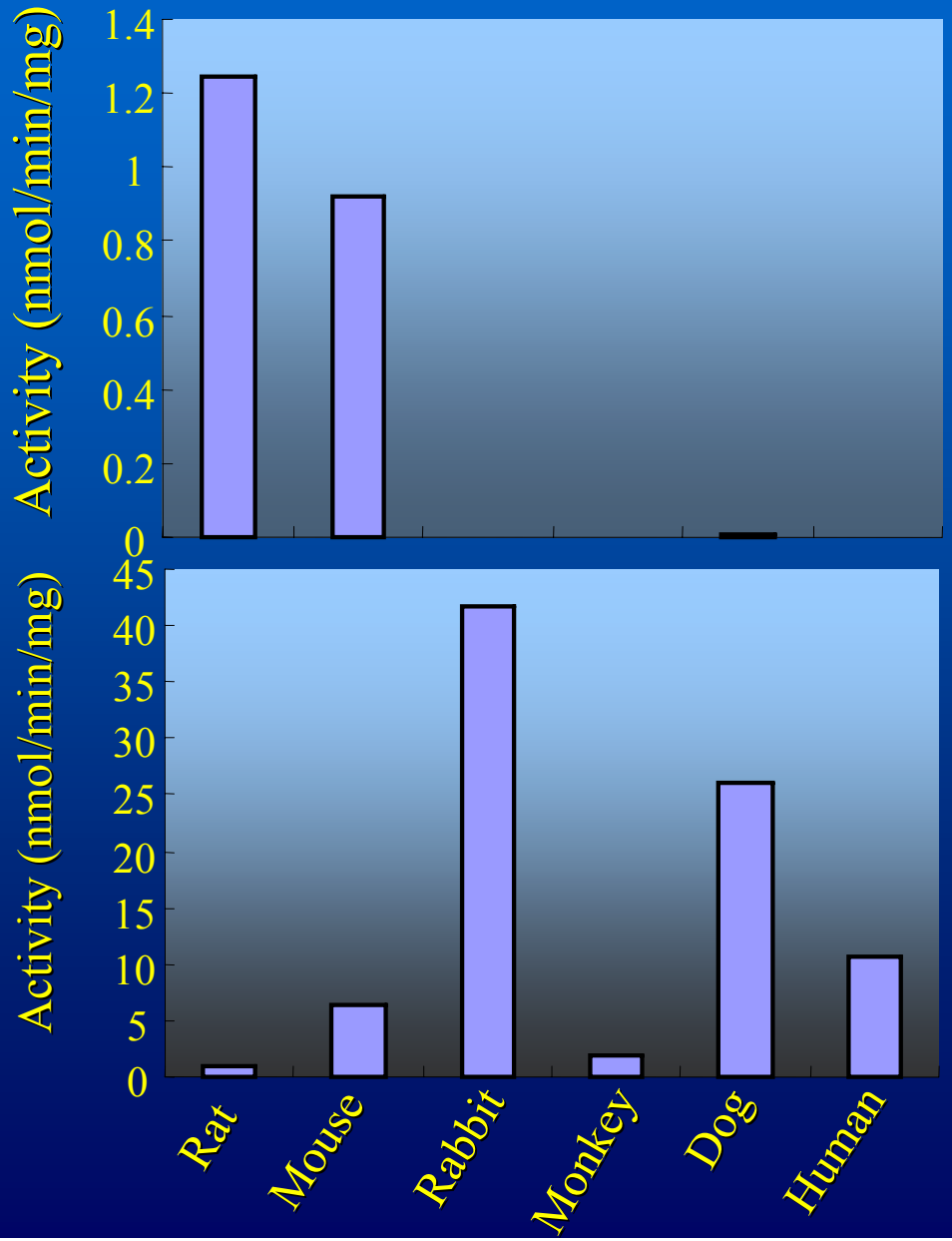




Hydrolysis of Olmesartan Medoxomil in Plasma



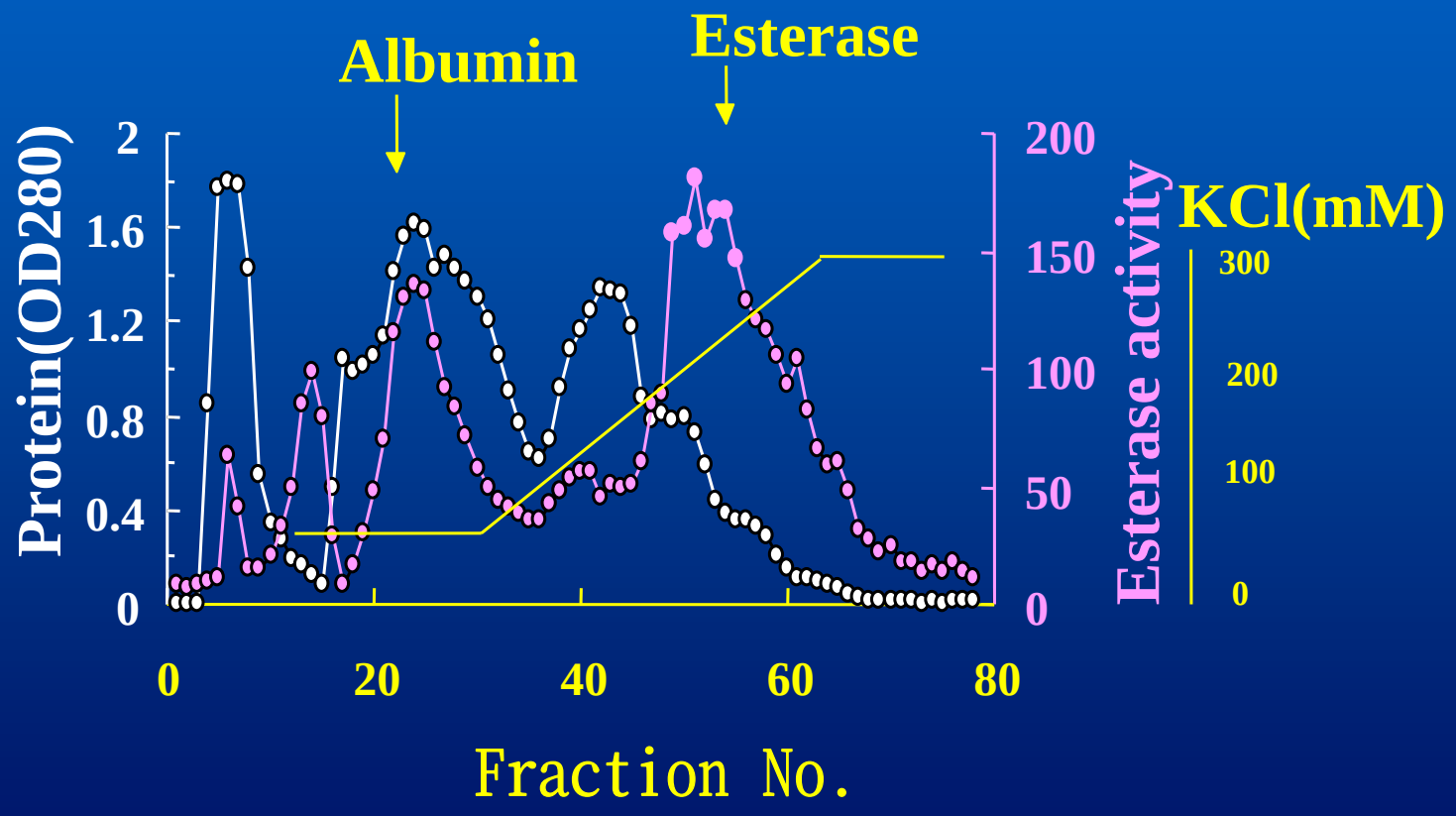
Comparison of Hydrolysis between Olmesartan Medoxomil and Temocapril in Plasma



Temocapril

Olmesartan Medoxomil

DEAE-Sephacel Chromatogram of Olmesartan Medoxomil-Esterase



Purification of Olmesartan Medoxomil-Esterase from Human Plasma

Substrate / Purification step	Olmesartan medoxomil Activity[1)]		Phenyl acetate Activity[2)]		Benzoyl choline Activity[3)]	
Plasma	13.0	(1)	0.027	(1)	13.42	(1)
Blue Sepharose	312.4	(24)	0.583	(22)	0.0	(0)
DEAE-Sephacel (1)	2457.0	(190)	3.067	(114)	0.0	(0)
DEAE-Sephacel (2)	5021.2	(389)	8.071	(299)	0.0	(0)

1)nmol/min/mg-protein 2) Δ OD/2min/mg-protein 3)nmol/min/mg-protein

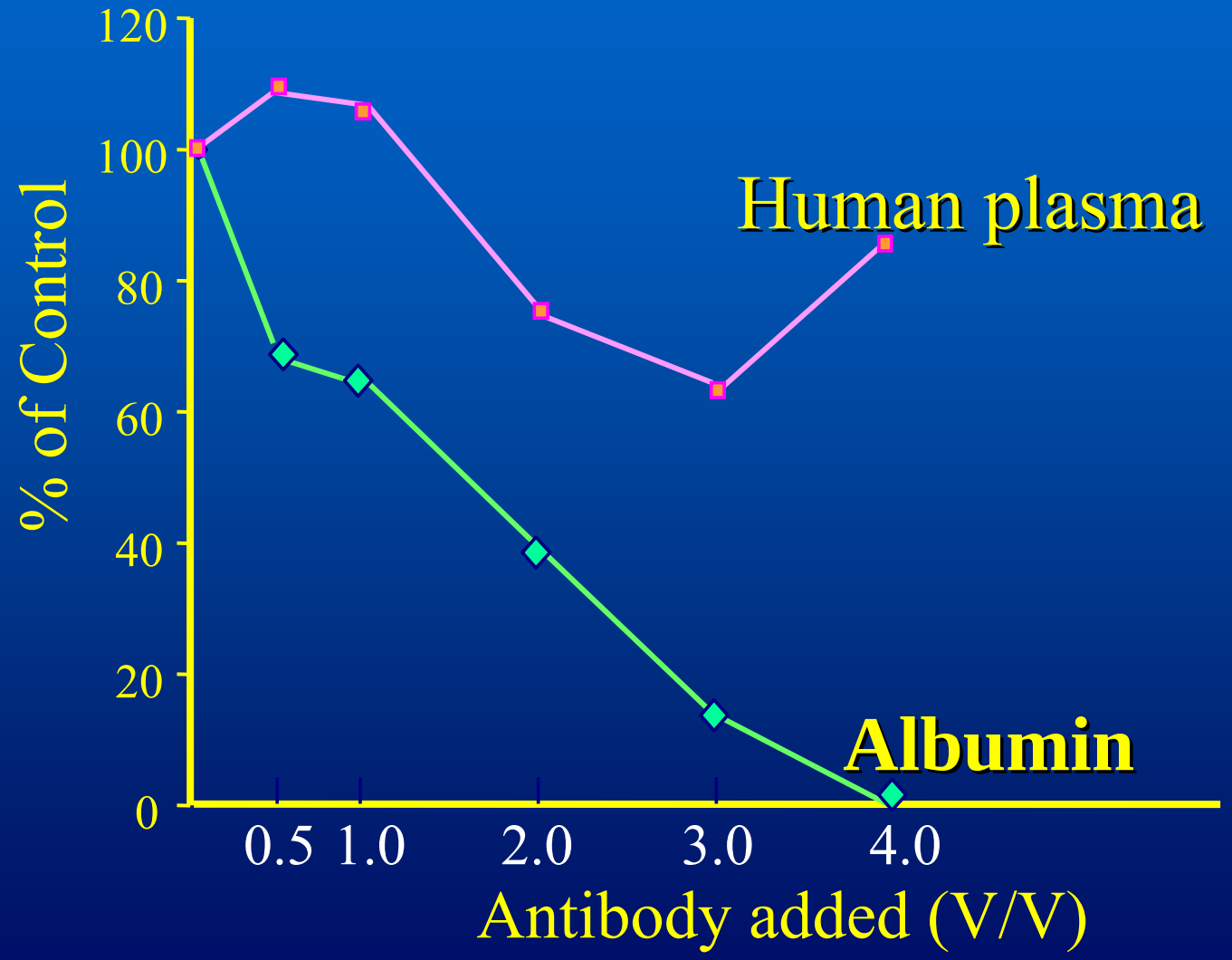
N-Terminal Amino Acid Sequence of Olmesartan Medoxomil-Esterase

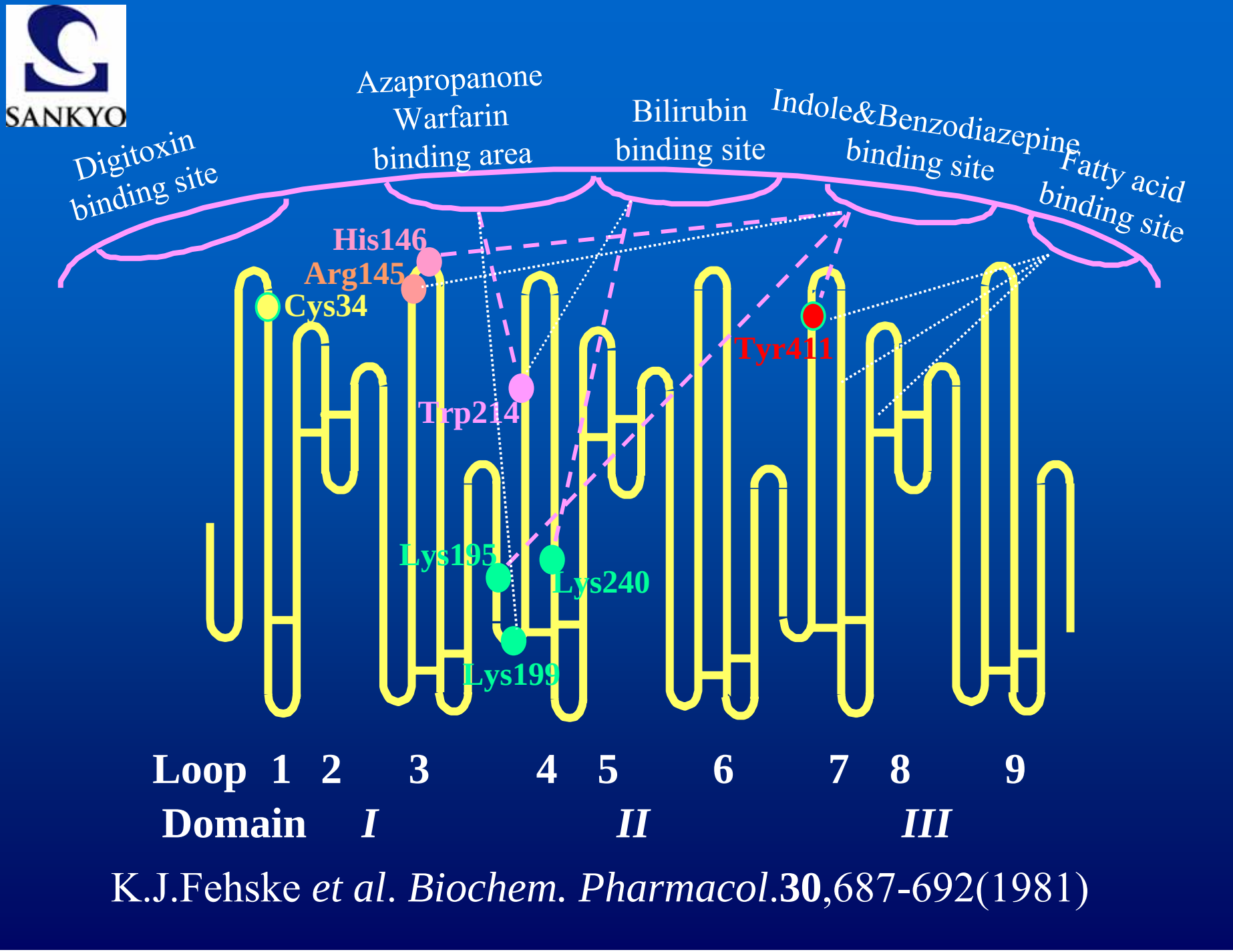
H_2N-Ala-Lys-Leu-Ile-Ala-Leu-Thr-Leu-Leu-Gly-Met-Gly-
Leu-Ala-Leu-Phe-Arg-Asn-His-Gln-

= Aryl esterase

SANKYO

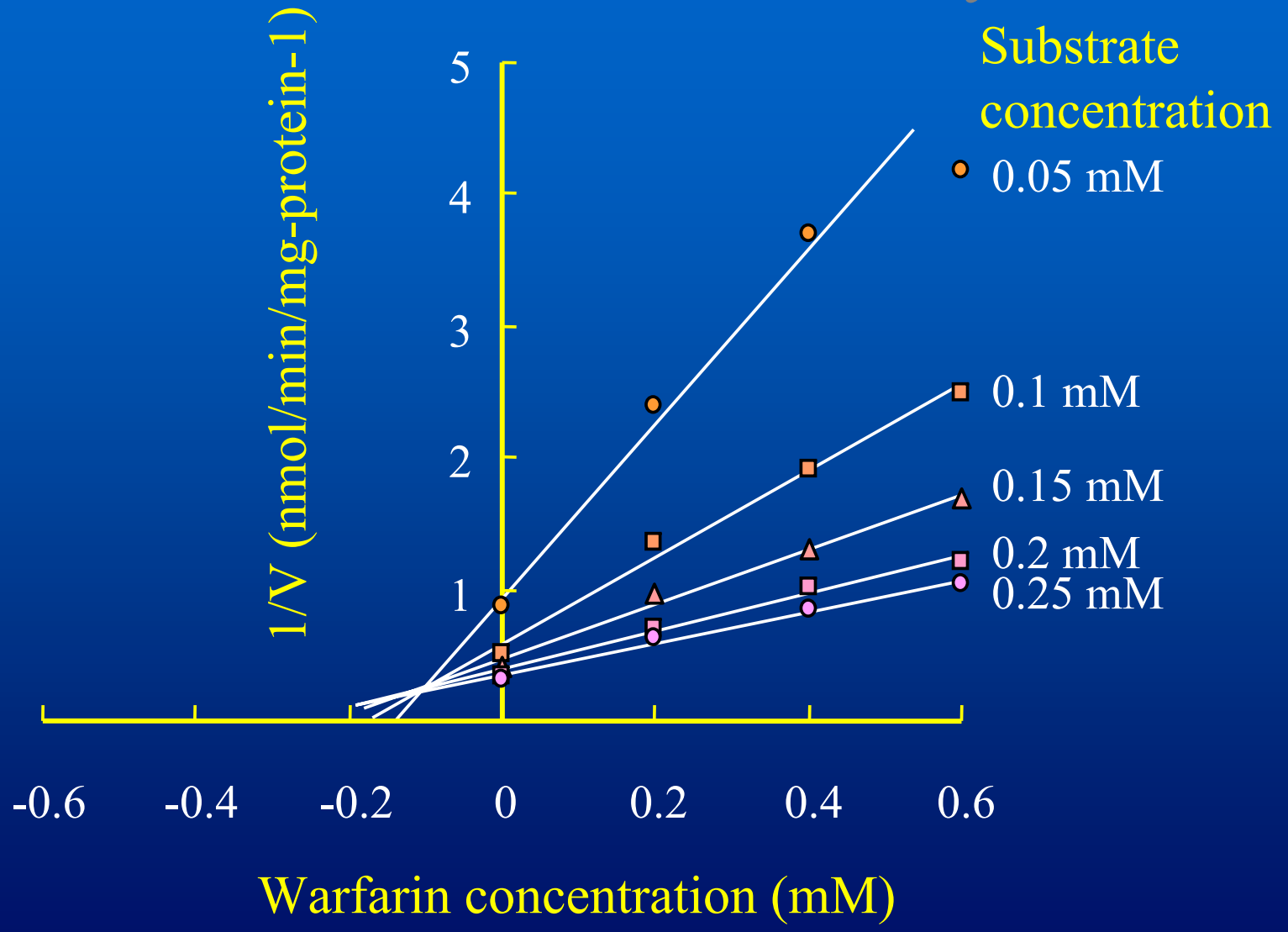
Inhibitory Effect of Anti-human Albumin on Hydrolysis of Olmesartan Medoxomil



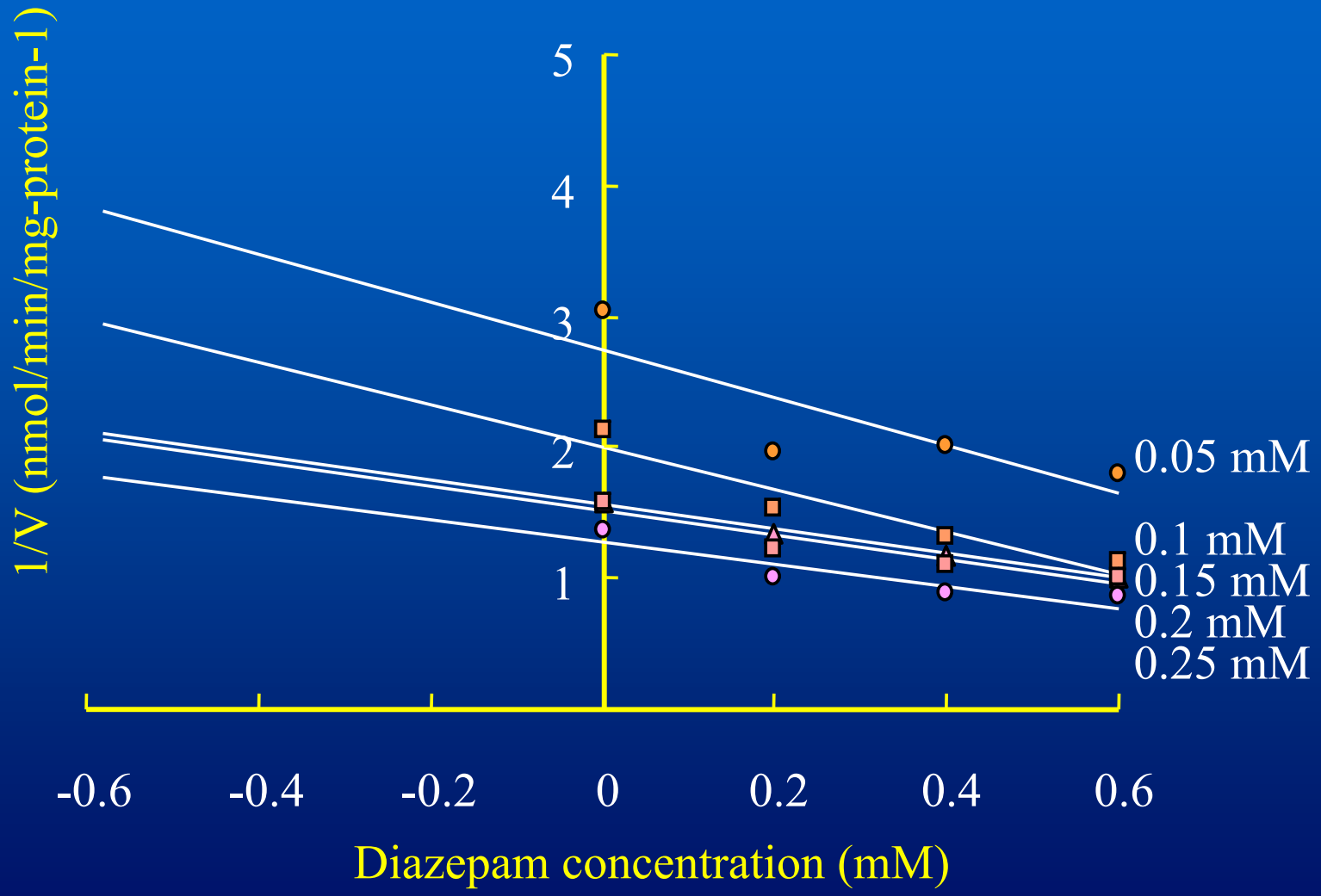


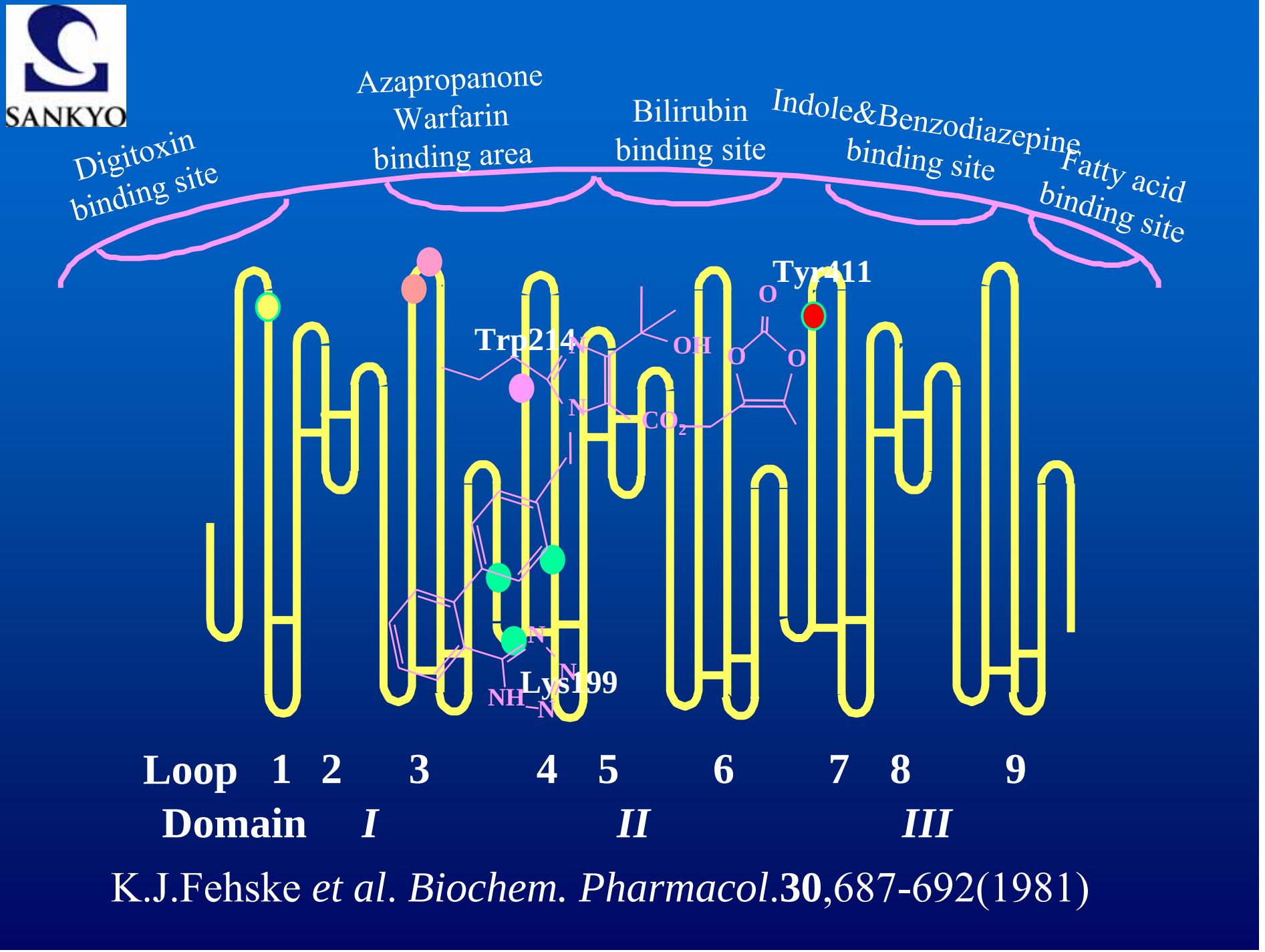
K.J.Fehske *et al. Biochem. Pharmacol.***30**,687-692(1981)

Effect of Warfarin on Hydrolysis of Olmesartan Medoxomil by Albumin



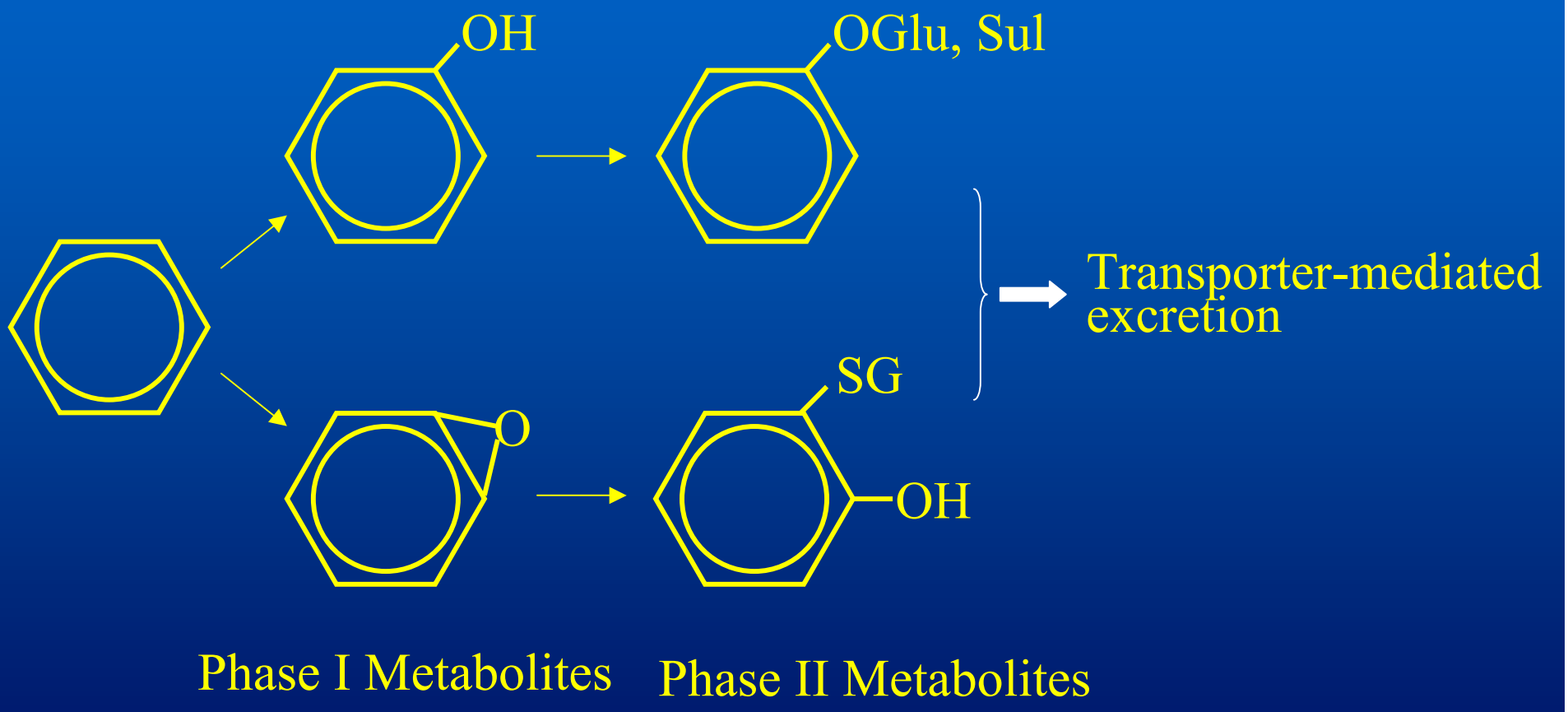
Effect of Diazepam on Hydrolysis of Olmesartan Medoxomil by Albumin





K.J.Fehske *et al. Biochem. Pharmacol.***30**,687-692(1981)

Principle in Drug Metabolism and Disposition

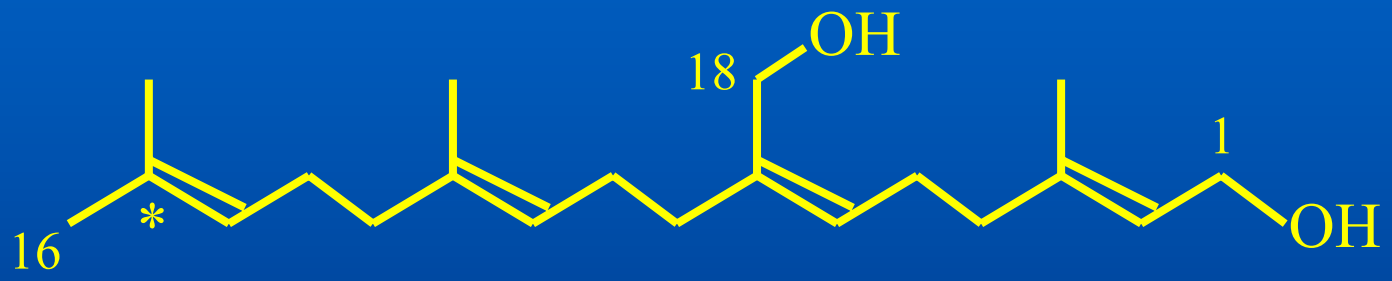


Two Exceptional Cases of Conjugation Reactions Disobeying the Principle

1. Metabolites more lipophilic than the parent drug

2. Monoglucuronide serving as the substrate for further glucuronidation

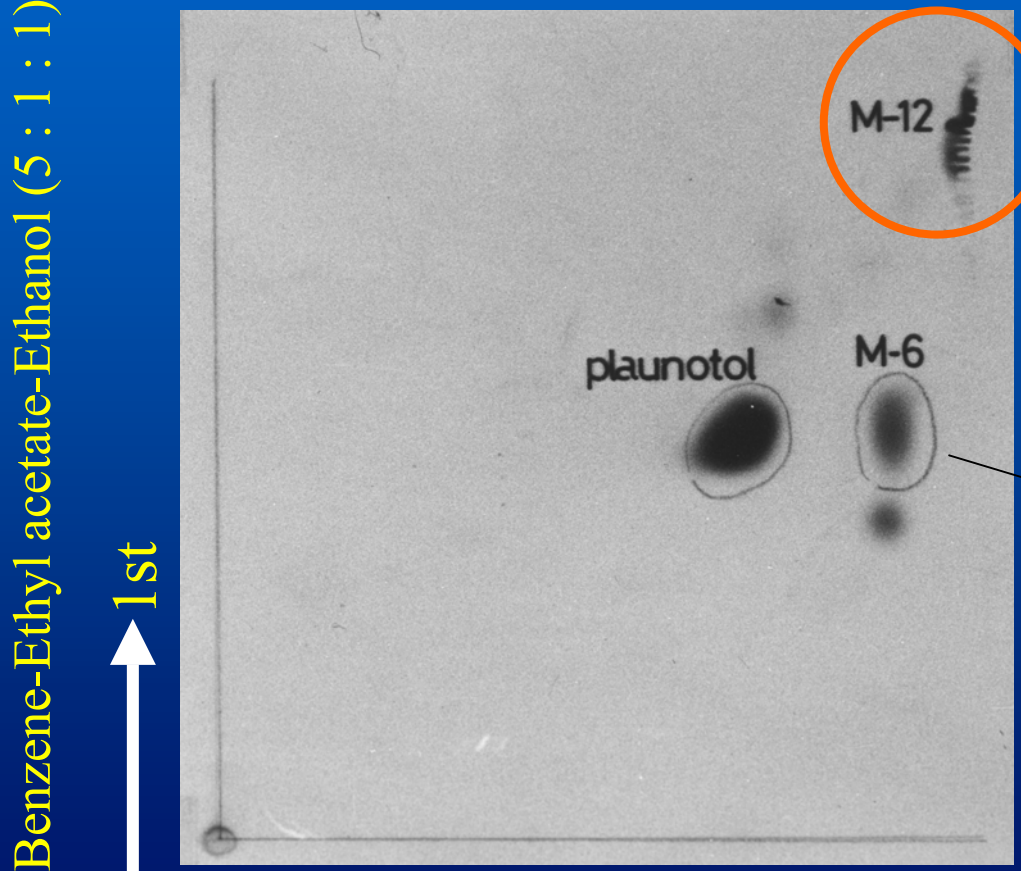
Plaunotol

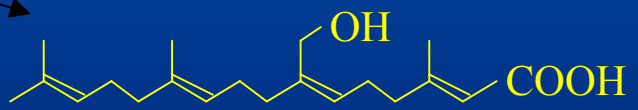


*:Position labeled with ^{14}C

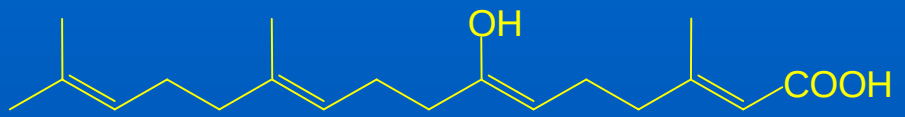
$C_{20}H_{34}O_2$

In Vitro Metabolites of Plaunotol Produced by Rat Liver Homogenate



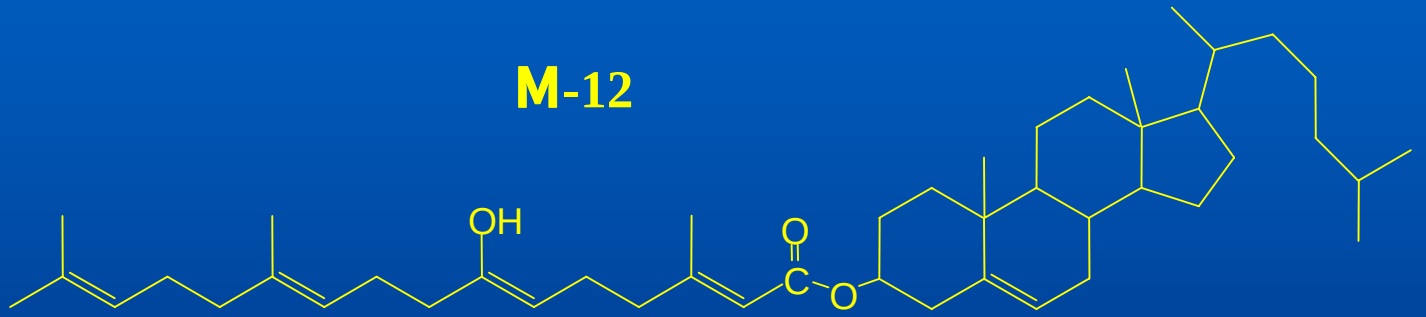


Speculated Chemical Structures of M-12



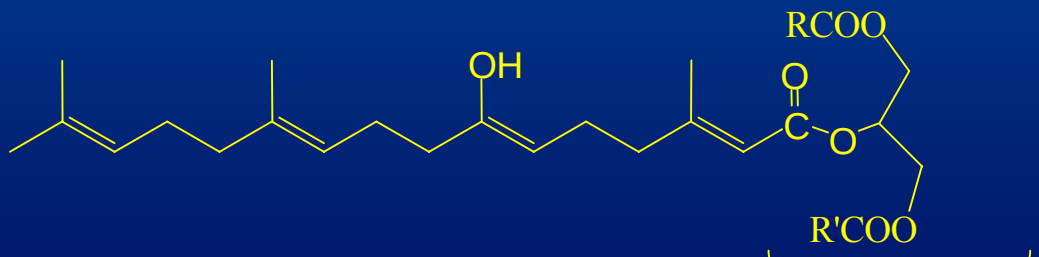
M-6: Major metabolite in plasma

M-12



Cholesterol ester of M-6

(Cholesterol moiety)

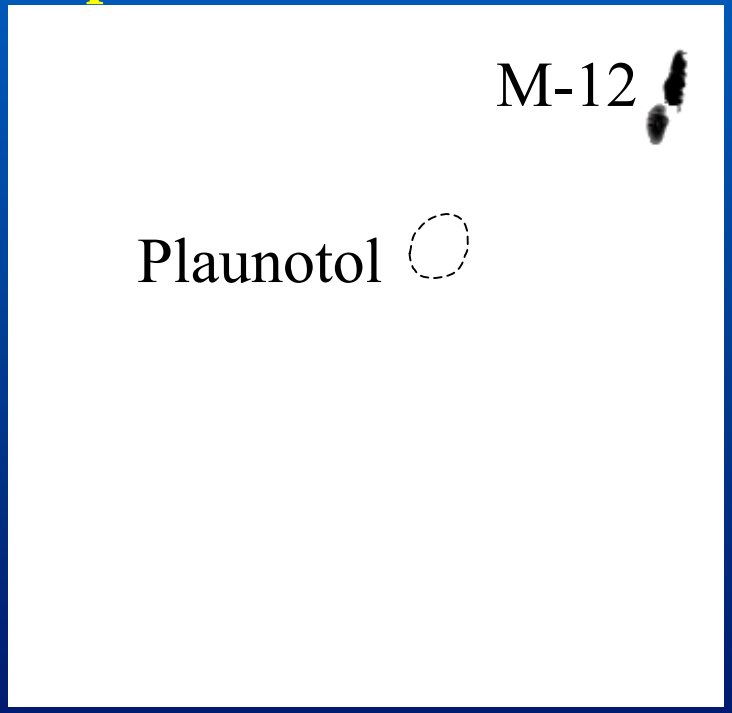


Triglycerides containing M-6

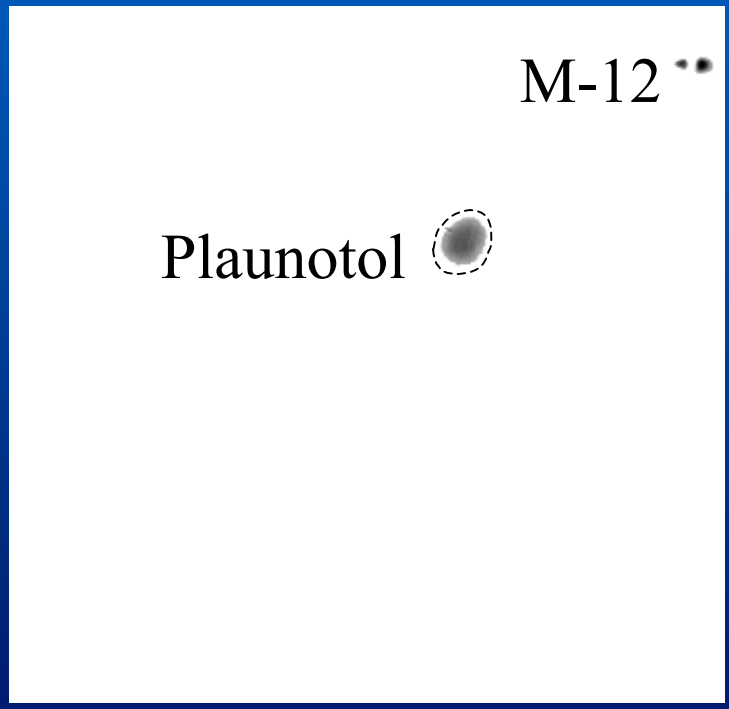
(Glycerol moiety)

Lipase- and Cholesterol Esterase- Treatments of Highly Lipid Soluble Metabolites of Plaunotol (M-12 *in vitro*, liver homogenates)

Treatment with Lipase



Treatment with Cholesterol Esterase



Benzene-Ethyl acetate-Ethanol (5 : 1 : 1)

1st

2nd

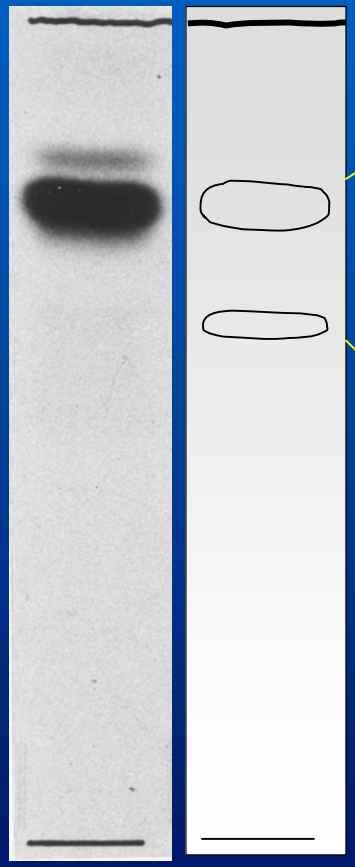
Isopropylether-Acetic acid (9 : 1)

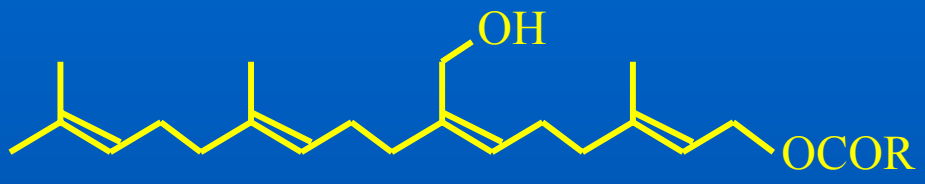
Identification of Plaunotol-1-fatty acid esters

TLC

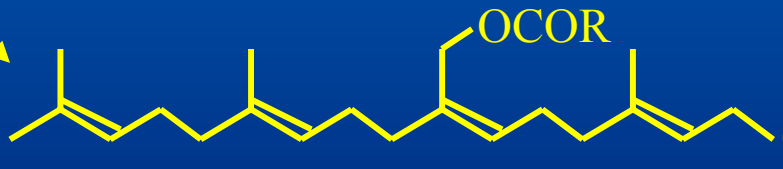
Detection

RI UV





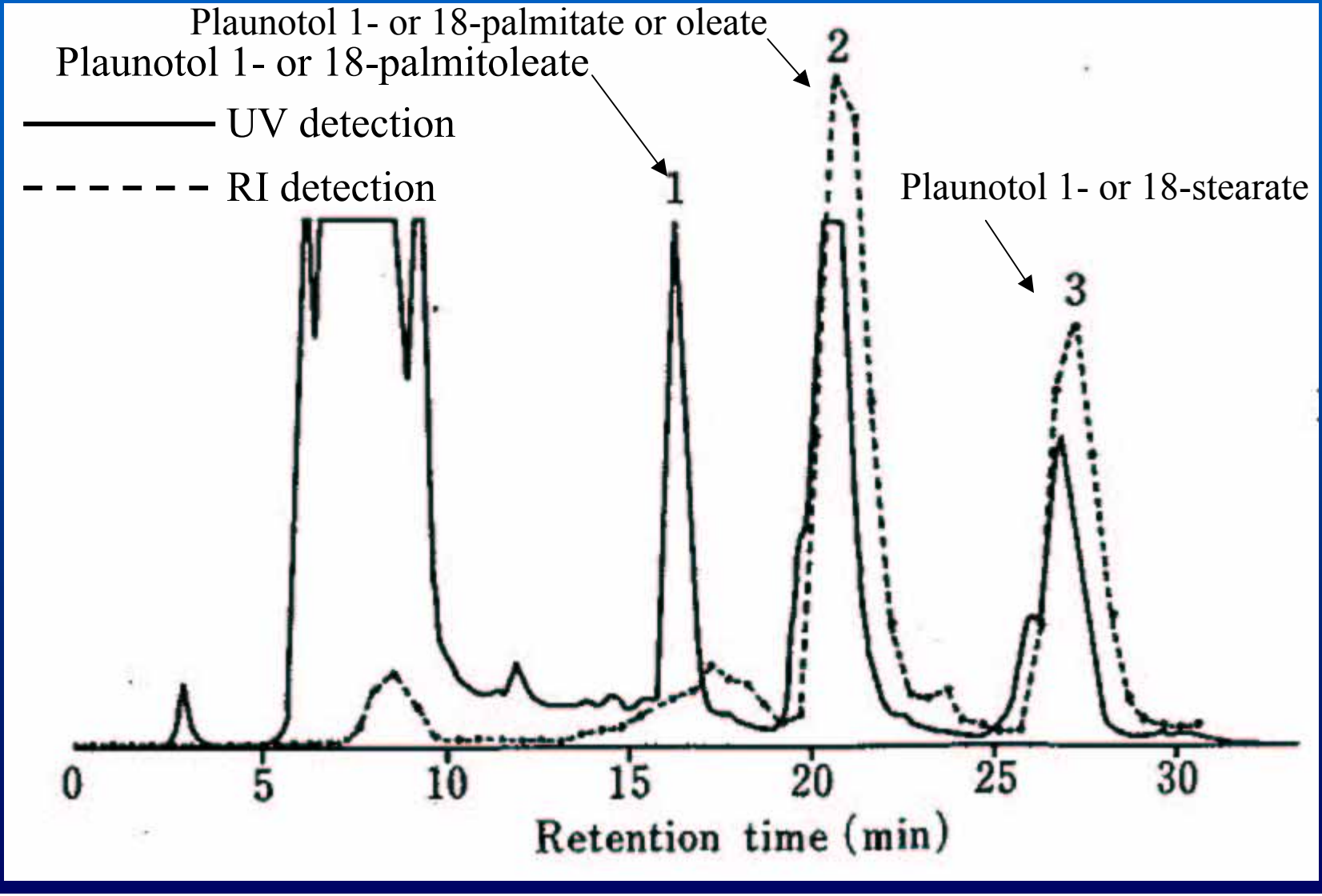
Plaunotol-1-palmitate
Plaunotol-1-stearate
Plaunotol-1-oleate



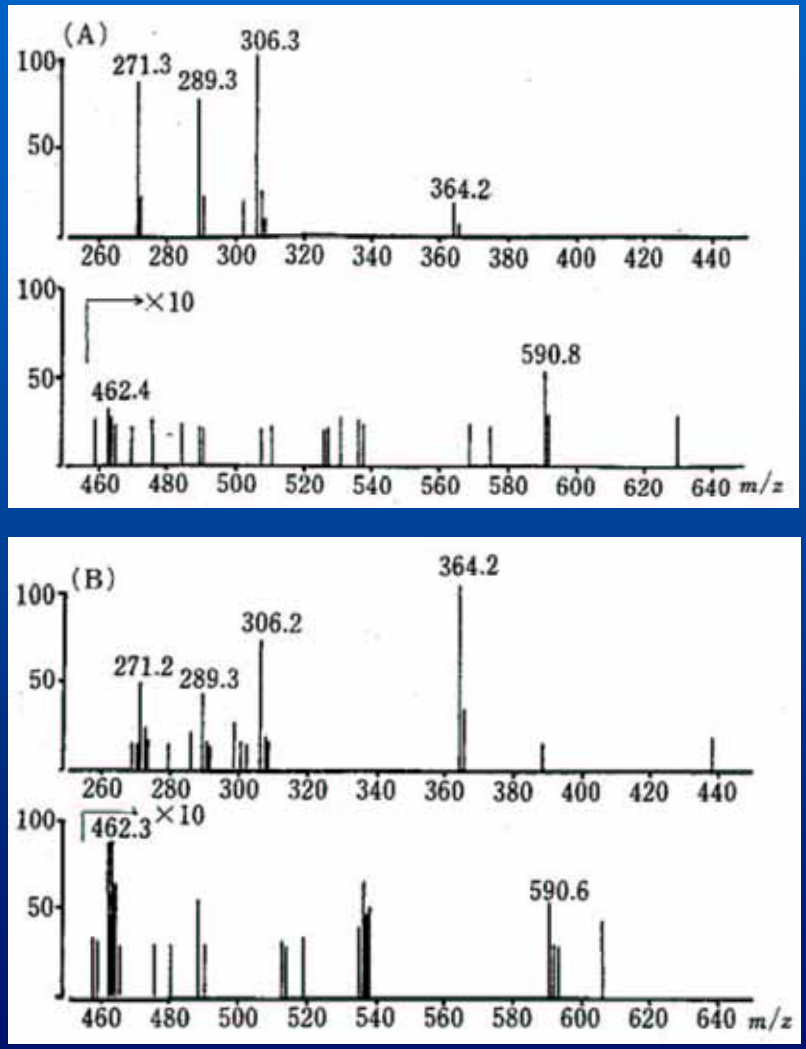
Plaunotol-18-palmitate
Plaunotol-18-stearate
Plaunotol-18-oleate

Hexane-Ethyl acetate (7 : 3)

Co-chromatogram of M-12 with Authentic Standards of Plaunotol Fatty Acid Esters



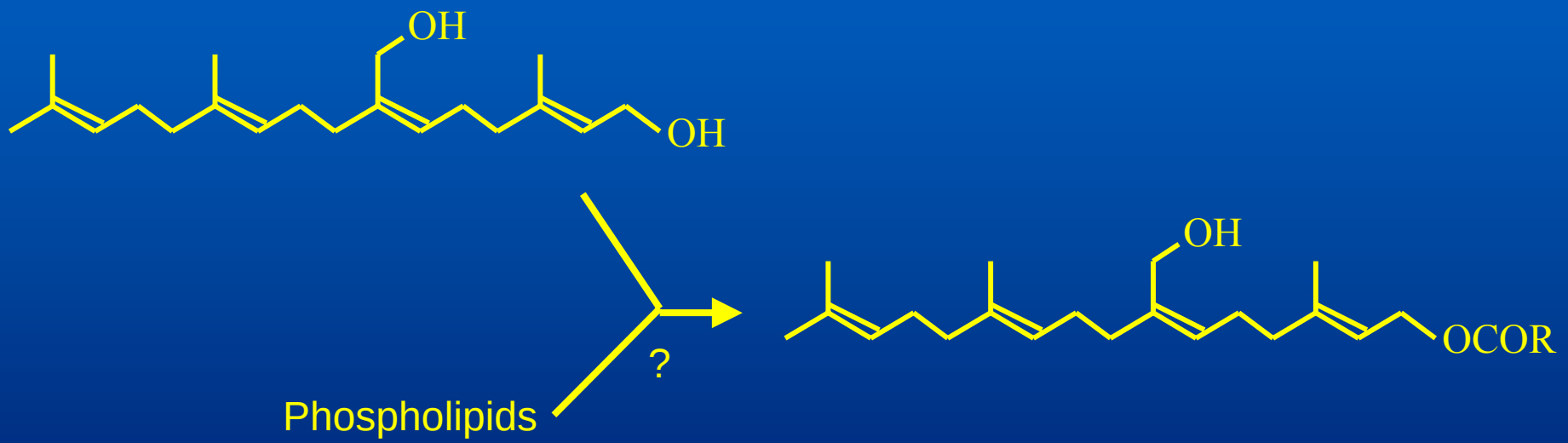
Identification of Plaunotol Stearate by GC/MS



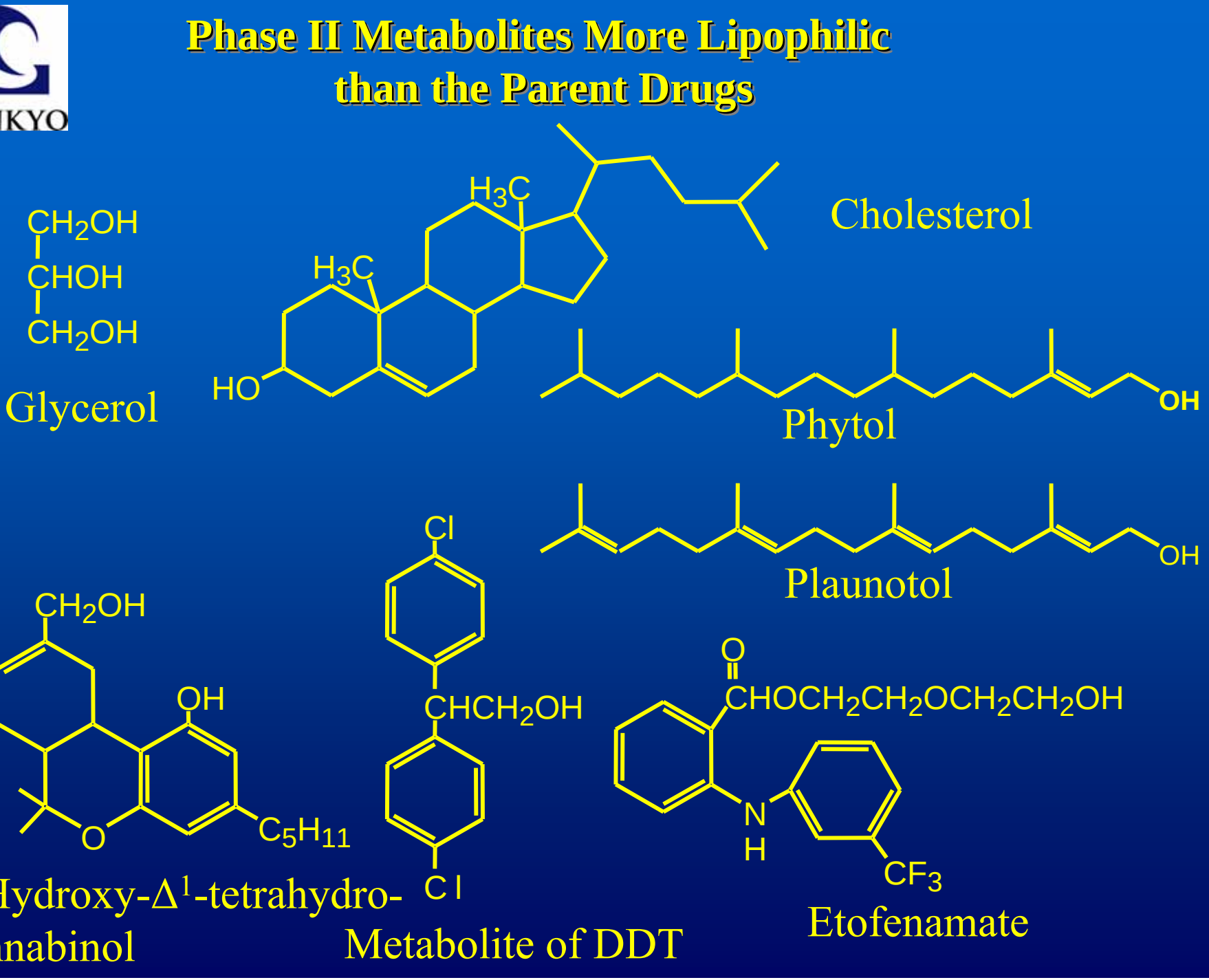
Synthetic authentic standard of plaunotol-1-stearate

Plaunotol-1-stearate produced *in vitro*

Plaunotol



Phase II Metabolites More Lipophilic than the Parent Drugs

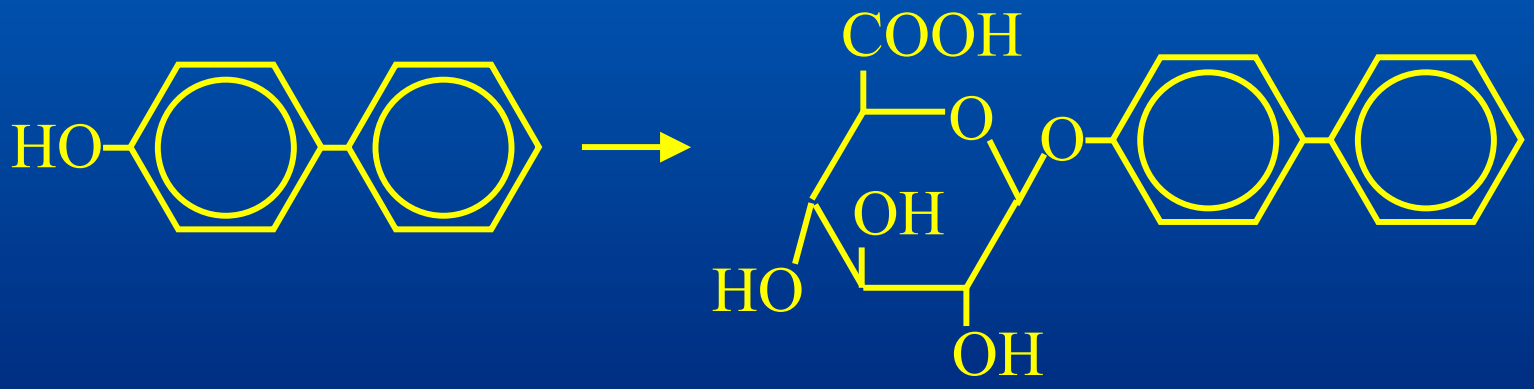


Two Exceptional Cases of Conjugation Reactions Disobeying the Principle

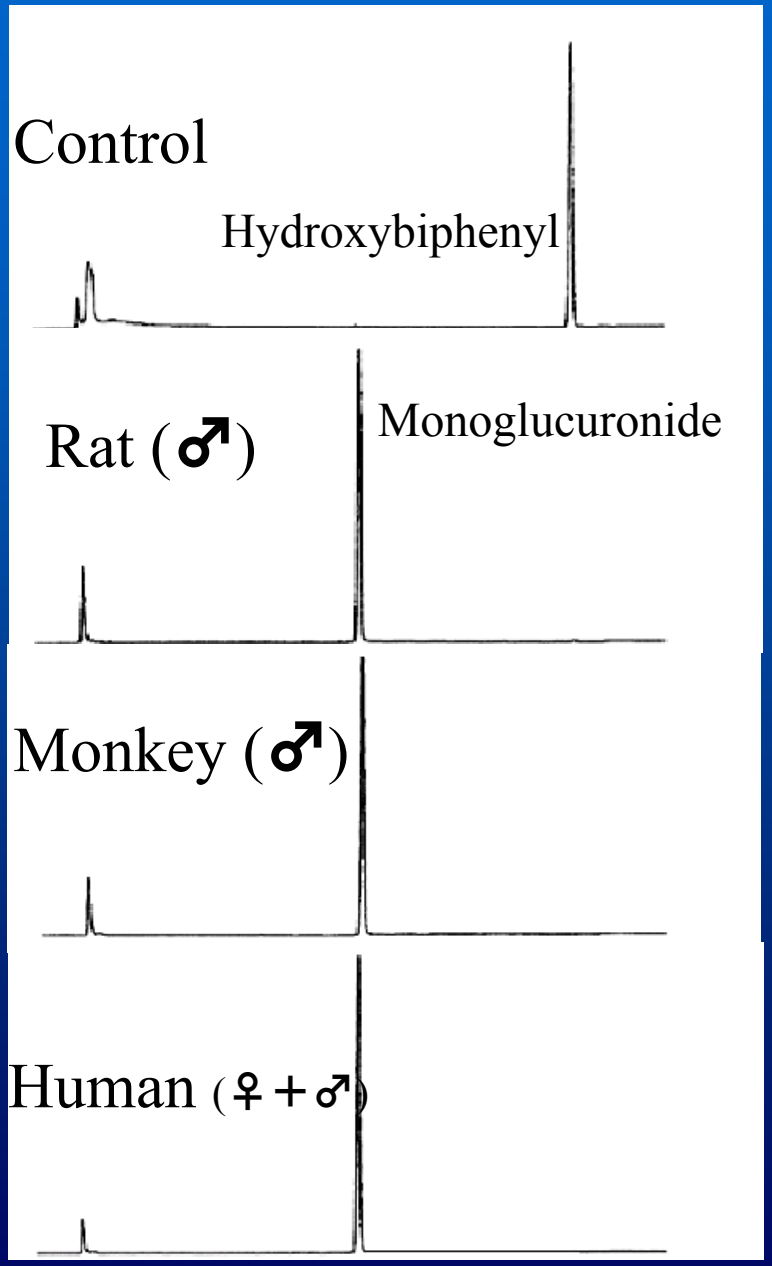
1. Metabolites more lipophilic than the parent drug

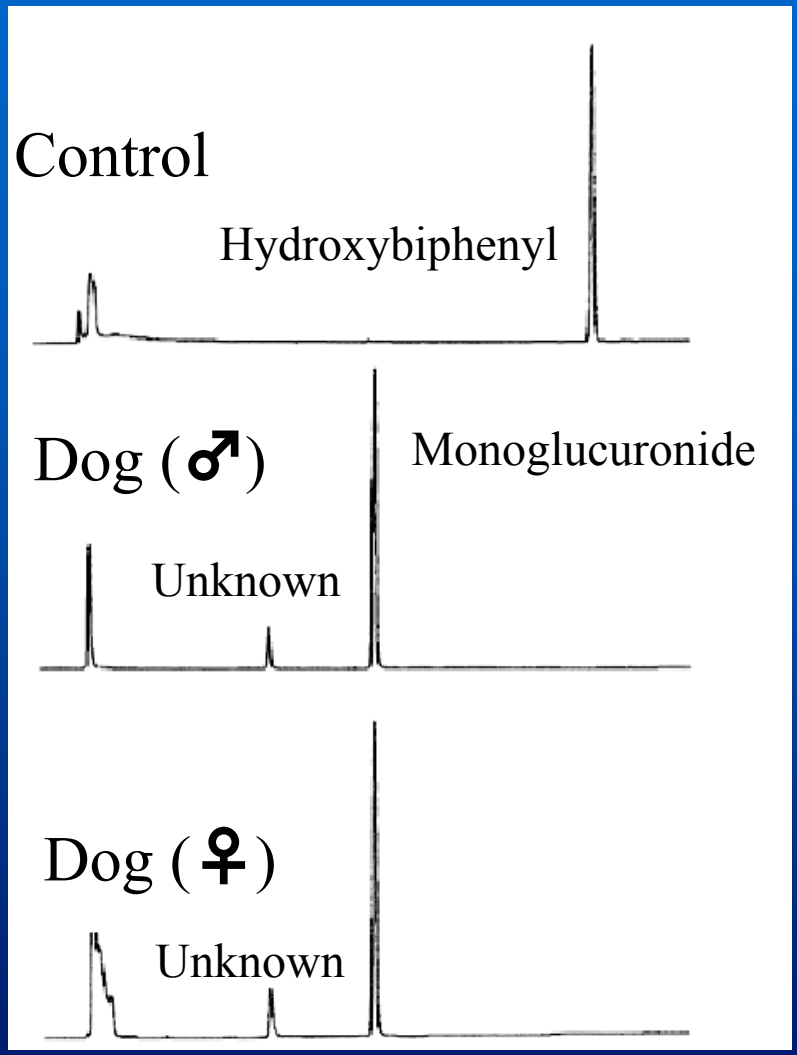
2. Monoglucuronide serving as the substrate for further glucuronidation

Formation of Diglucuronide of Hydroxybiphenyl



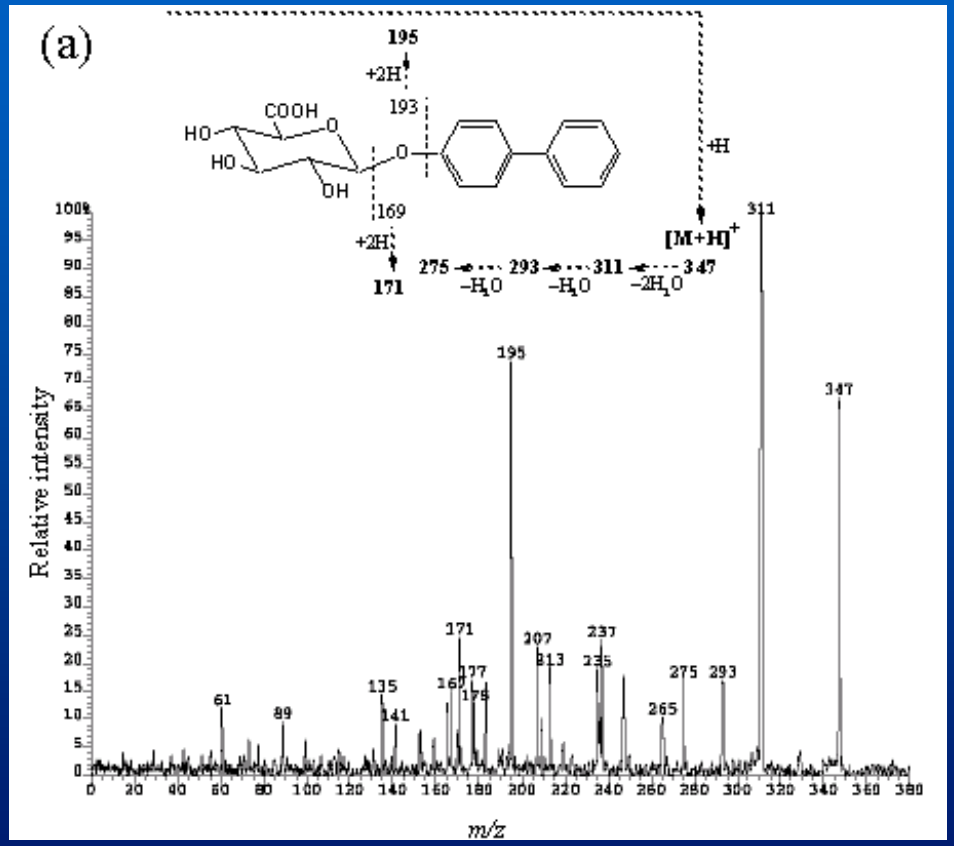
Glucuronidation Products of Hydroxybiphenyl

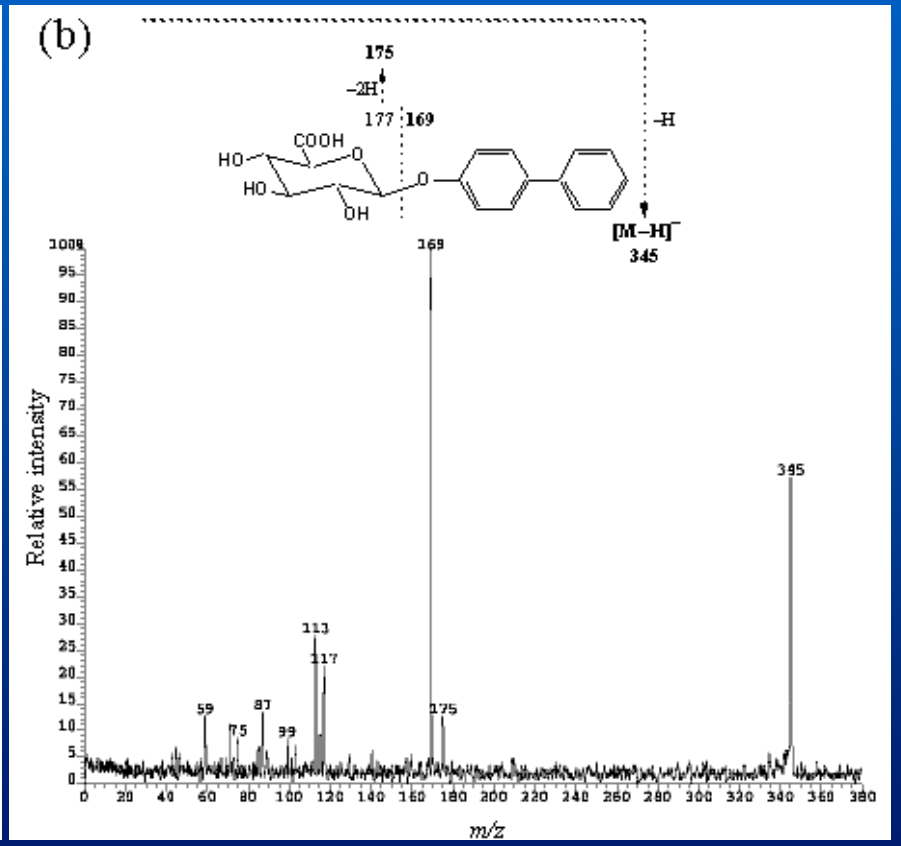




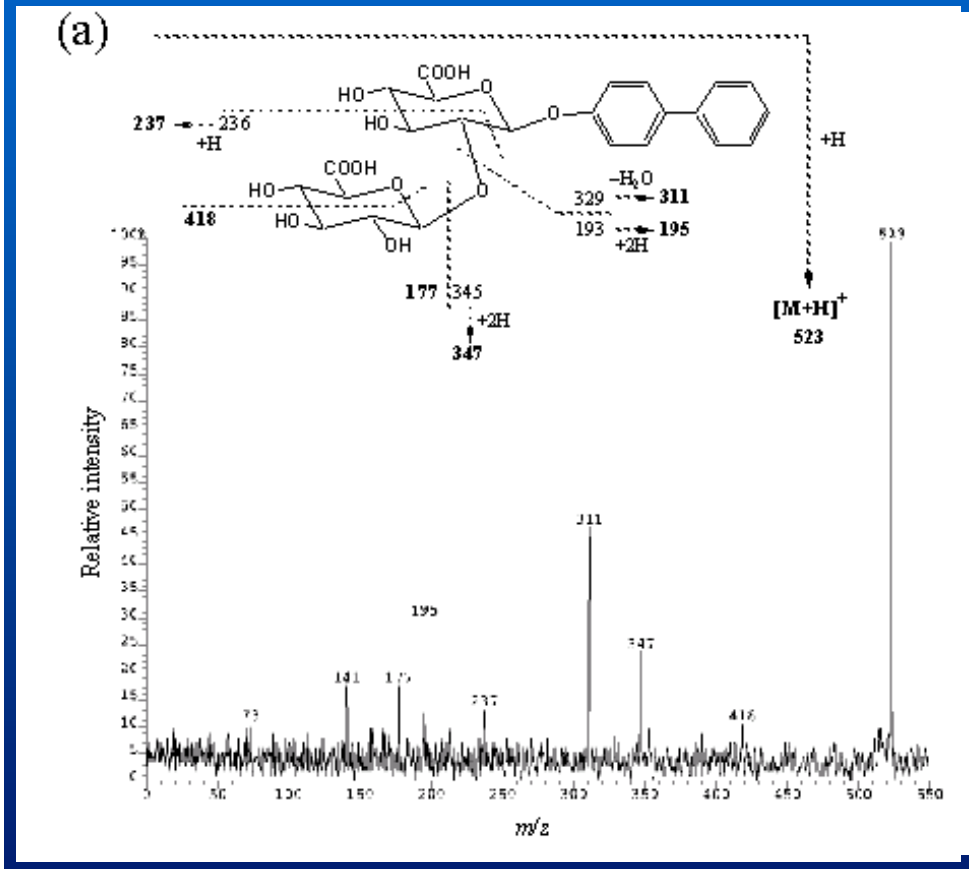
Liver microsomes (1 mg/ml)
Hydroxybiphenyl (100 μM)
UDPGA(10mM)
6 hr

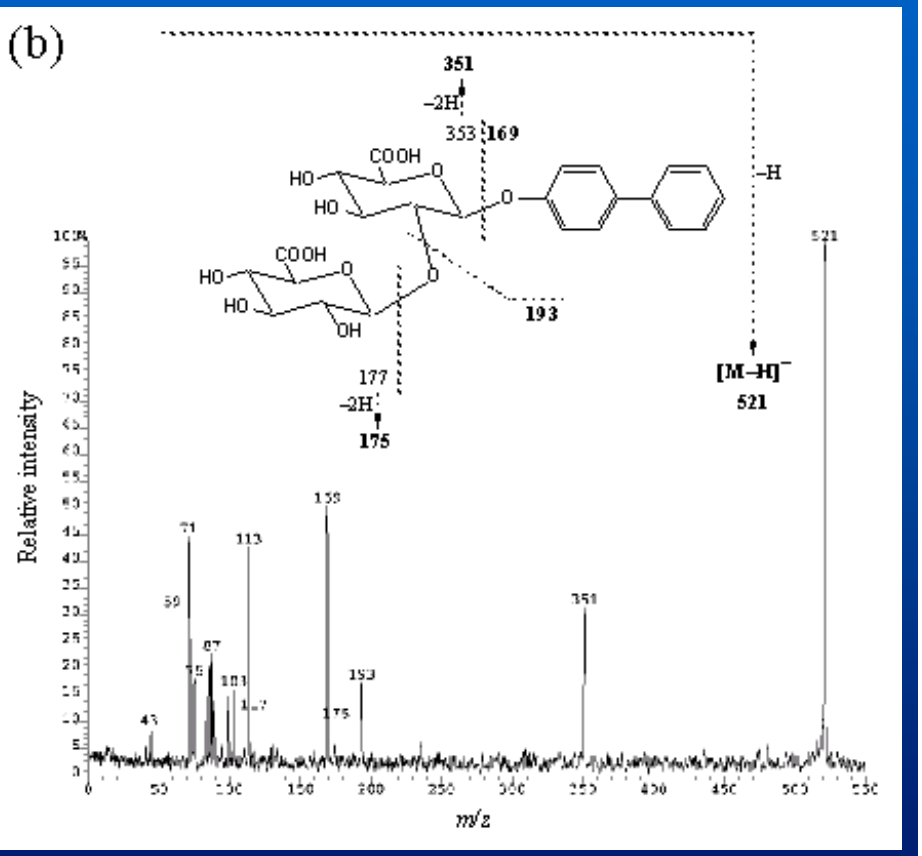
Structure Elucidation of Monoglucuronide by MS/MS



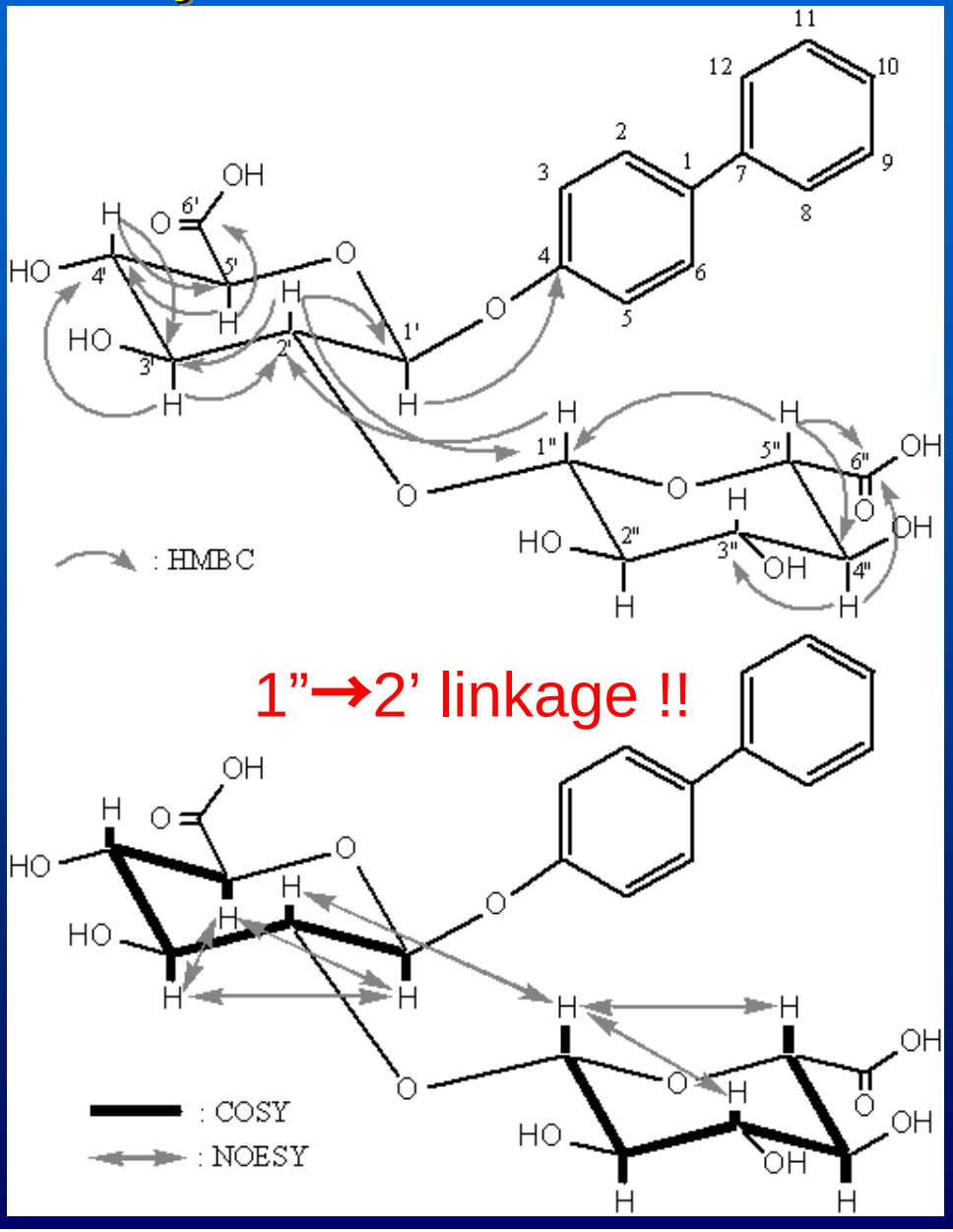


Structure Elucidation of Tandem Diglucuronide by MS/MS

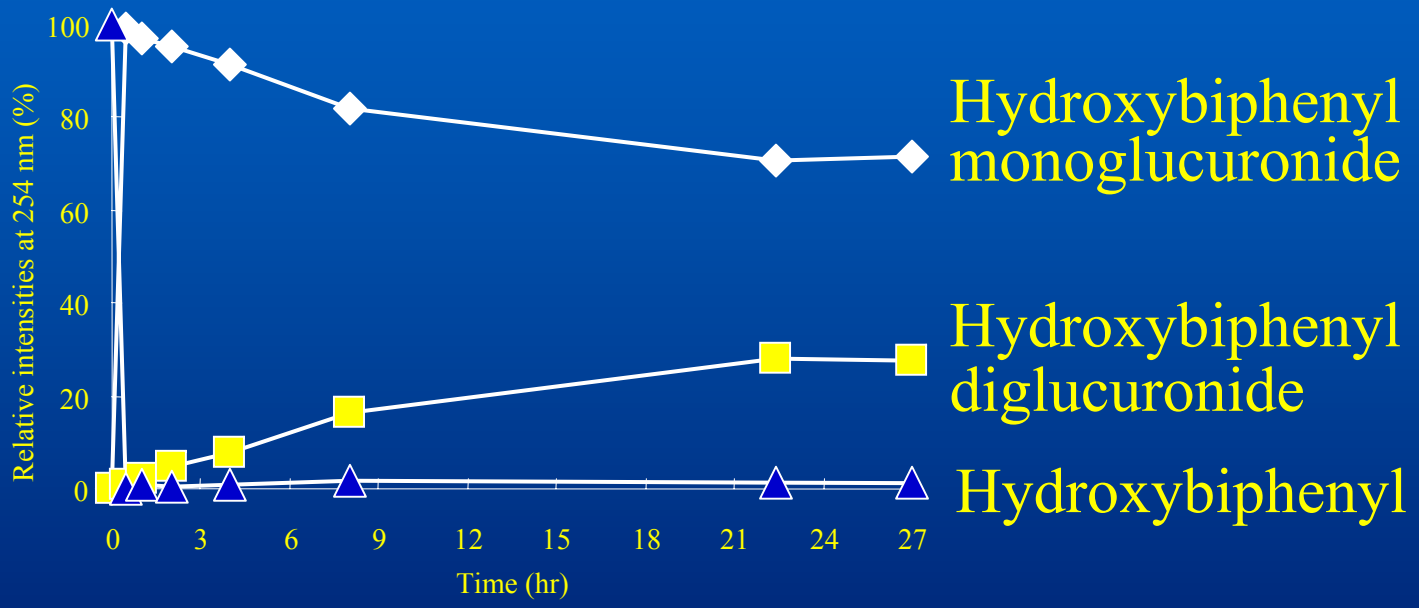




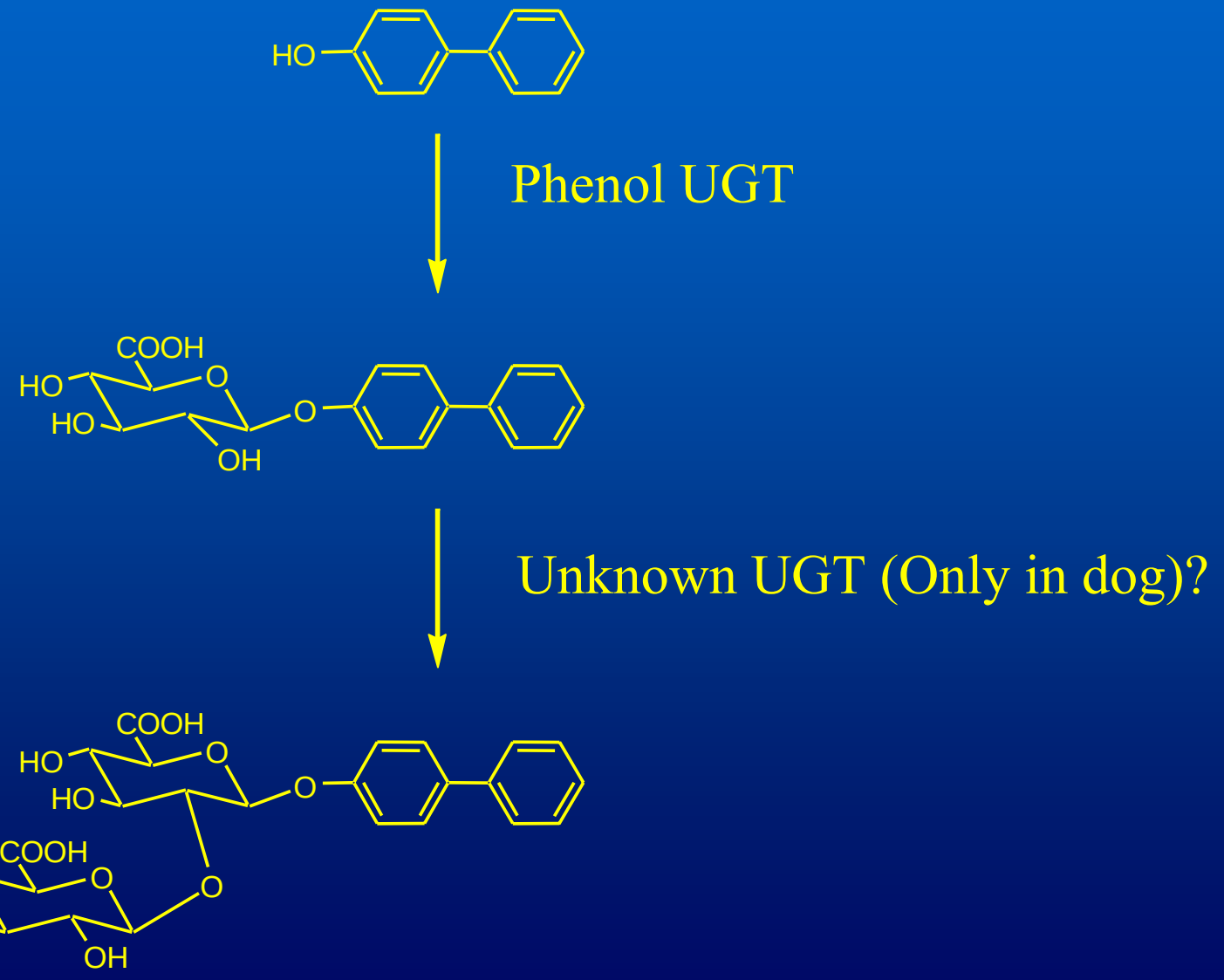
Structure Elucidation of Tandem Diglucuronide by Two-dimensional NMR



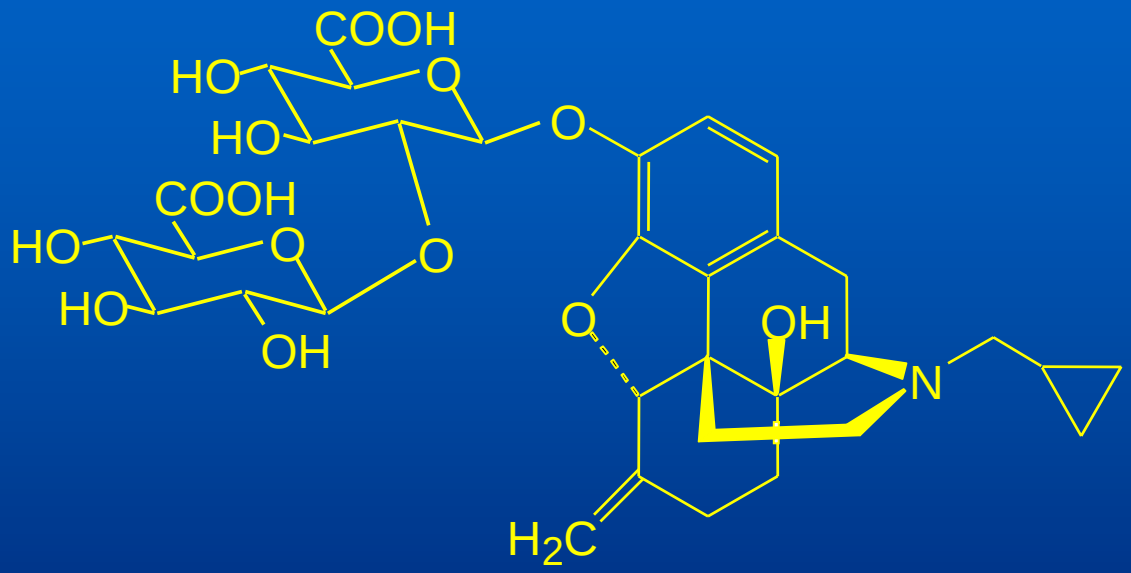
Sequential Production of Diglucuronide



Pathway for Production of Hydroxybiphenyl Diglucuronide



Nalmefene Diglucuronide Only in Dog Urine



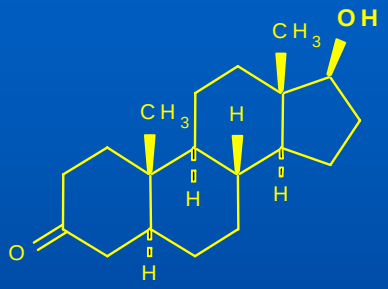
Nalmefene diglucuronide

1) Dixon, R., *et al. Pharm. Res.*, **6**: 28-32 (1989)
2) Murthy, S.S., *et al. Xenobiotica*, **26**: 779-792 (1996)

Formation of Diglucuronides of Endogenous Steroids

Androsterone

(MW: 290)



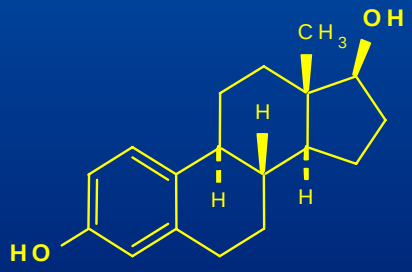
Dihyderotestosterone

(MW: 290)

Testosterone

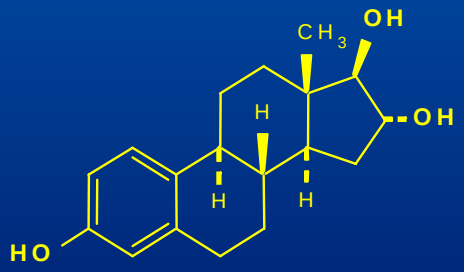
(MW: 288)

Diglucuronides?



Estradiol

(MW: 272)



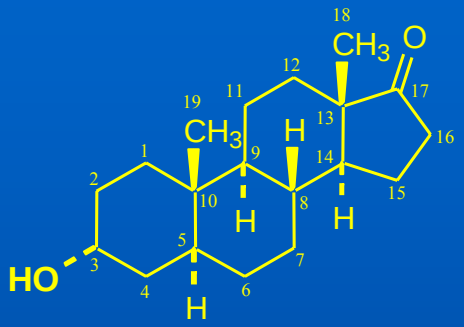
Estriol

(MW: 288)

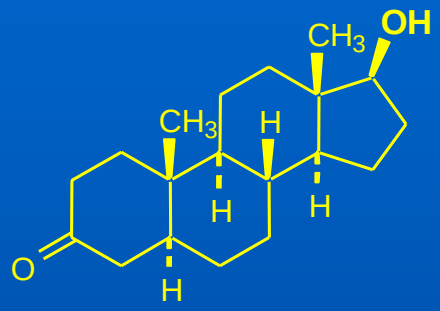
Estrone

(MW: 270)

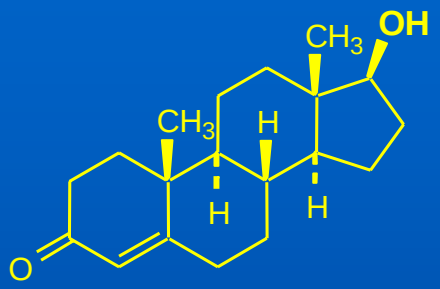
UGT Assay of Endogenous Steroids for Multiple Glucuronidation



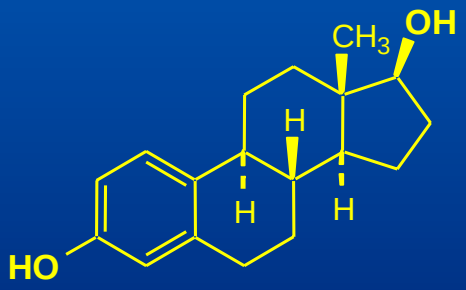
Androsterone
(MW: 290)



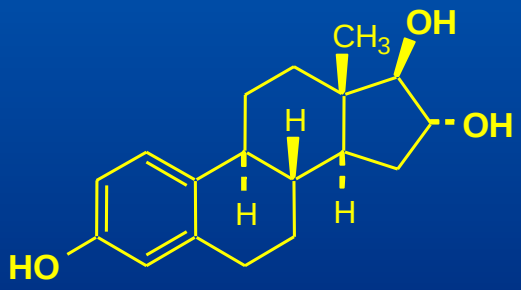
Dihyderotestosterone
(MW: 290)



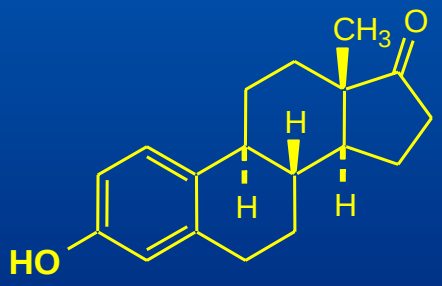
Testosterone
(MW: 288)



Estradiol
(MW: 272)



Estriol
(MW: 288)

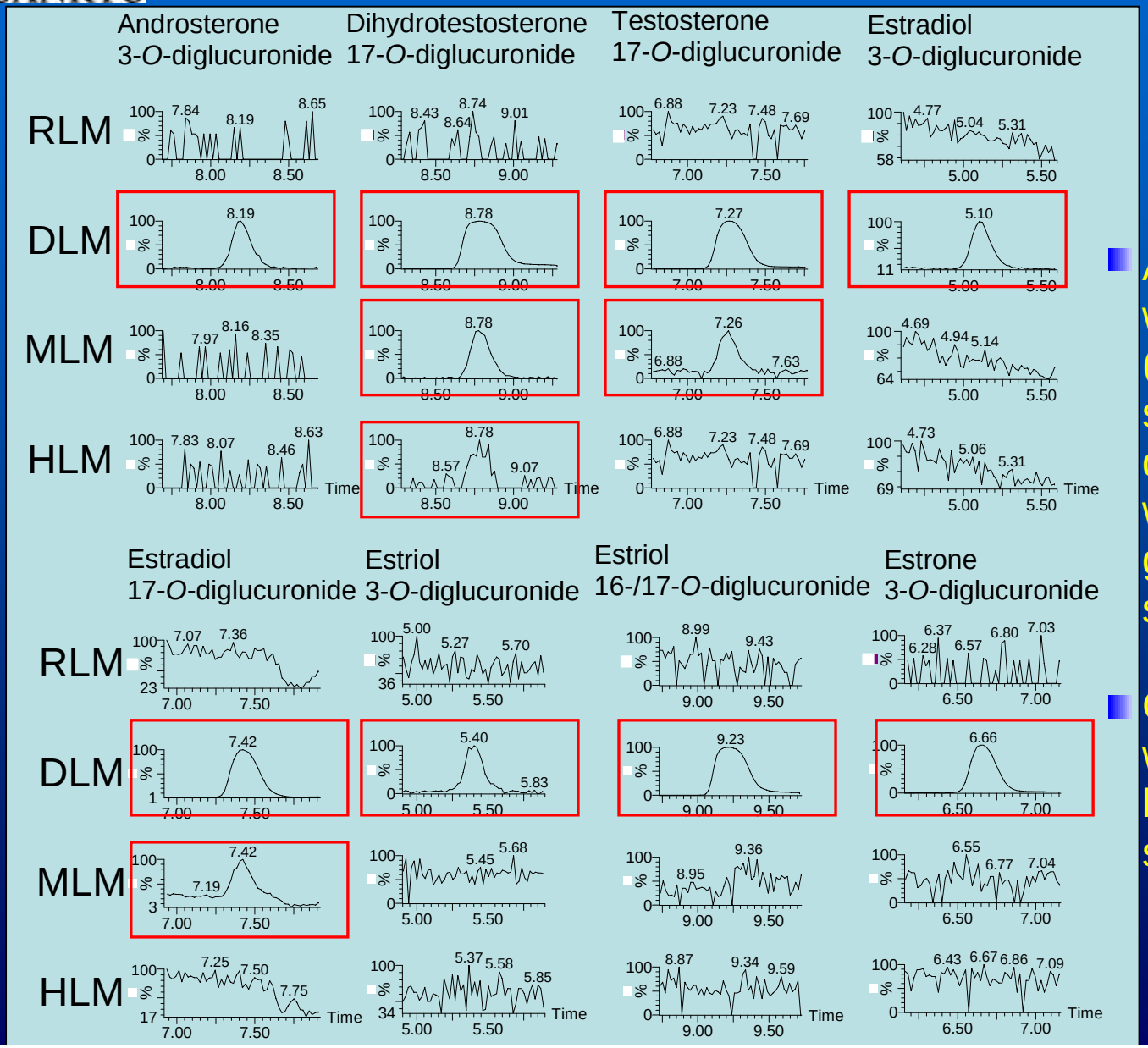


Estrone
(MW: 270)

Steroid substrate (1 mM)
Liver microsomes (2 mg protein/ml) treated with alamethicin (50 μg/mg protein)
Magnesium chloride (10 mM)
D-saccharic acid 1,4-lactone (5 mM)
UDPGA (5 mM)
in 100 mM sodium phosphate buffer (pH 7.4)

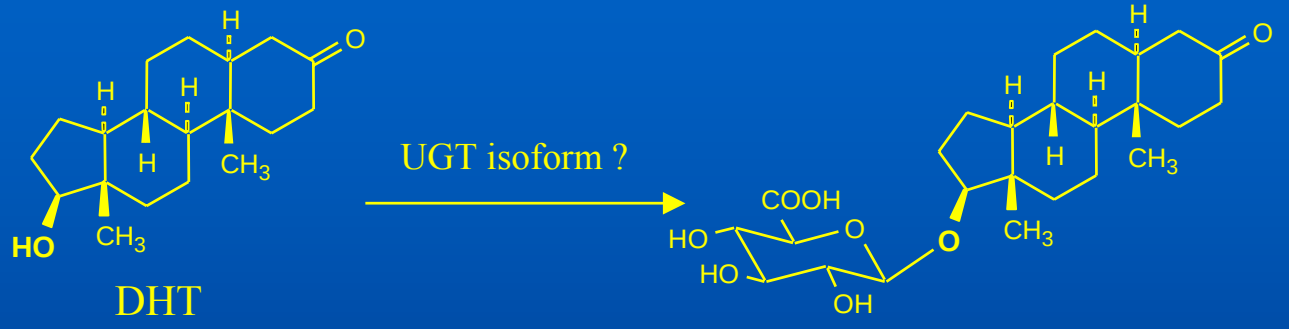
→incubated for 2 h at 37ºC
→LC/MS analysis

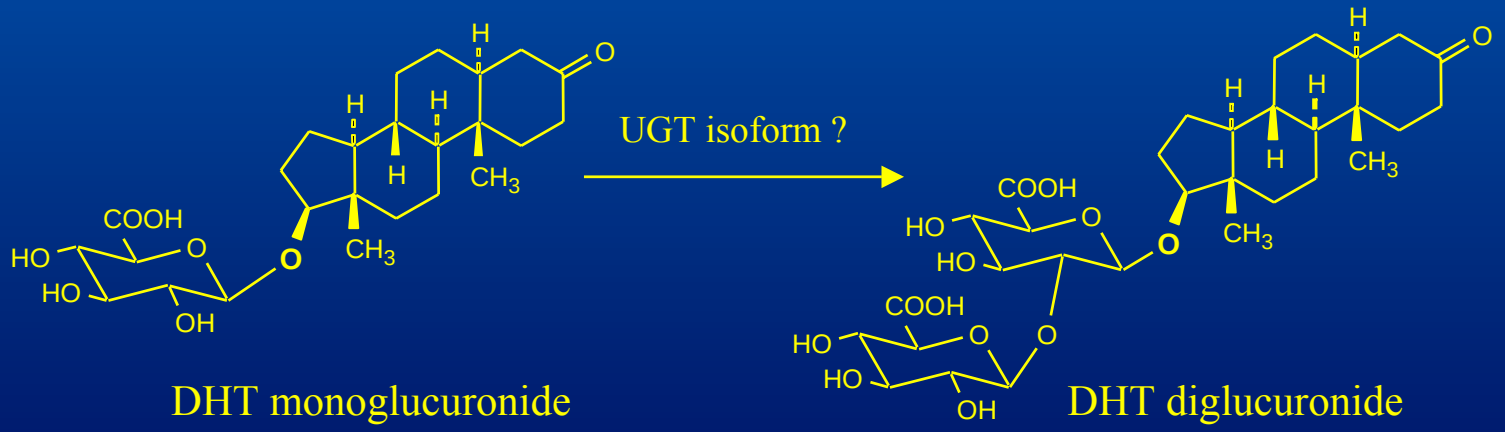
Detection of Steroid Diglucuronides
-Reconstructed Ion Chromatograms-



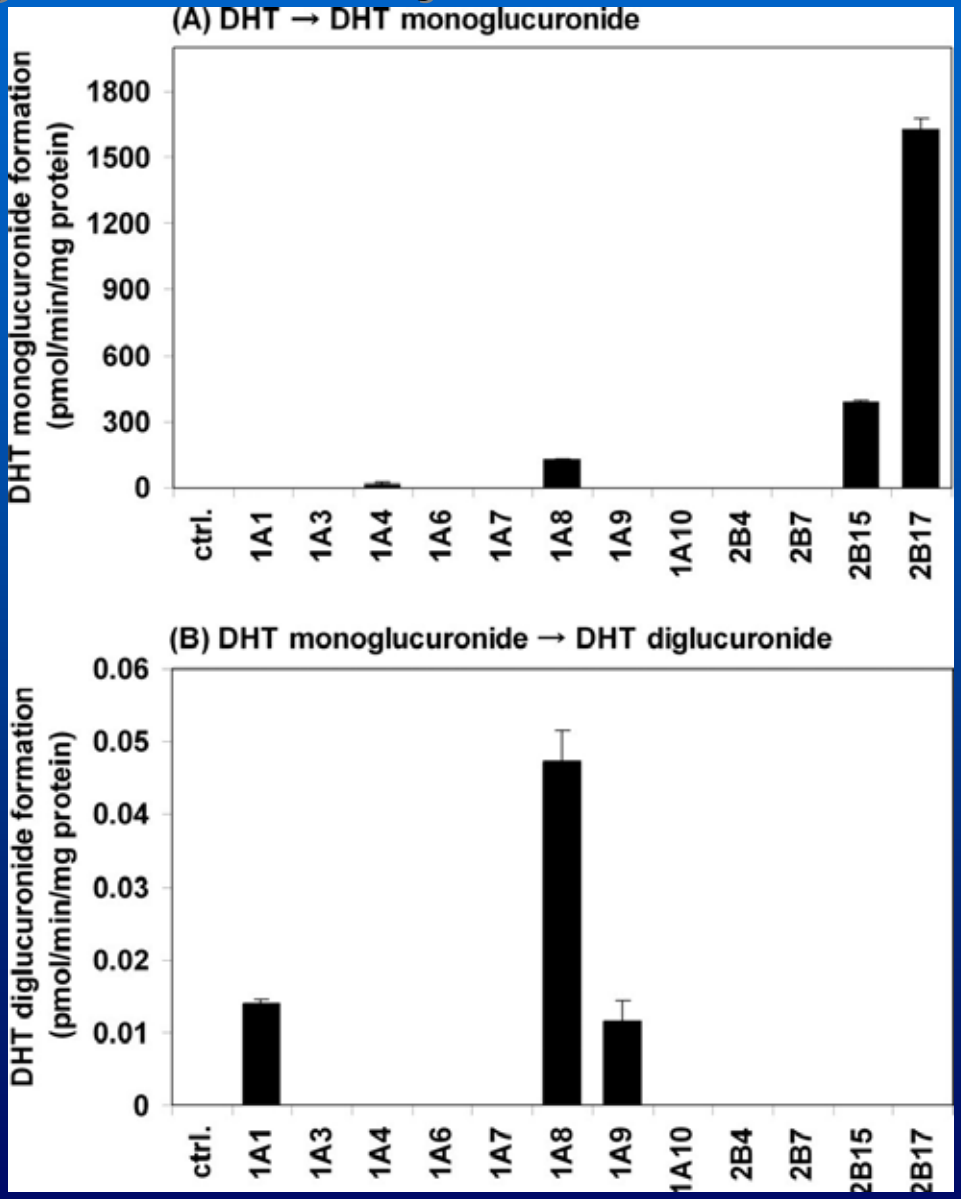
- All substrates tested were converted (approx.2-10%) to structurally novel diglucuronides, where two glucuronosyl groups are bound to a single hydroxyl group.
- Chemical structures were characterized by tandem mass spectrometry.

Characterization of Human UGT Isoform Responsible for Dihydrotestosterone Diglucuronidation

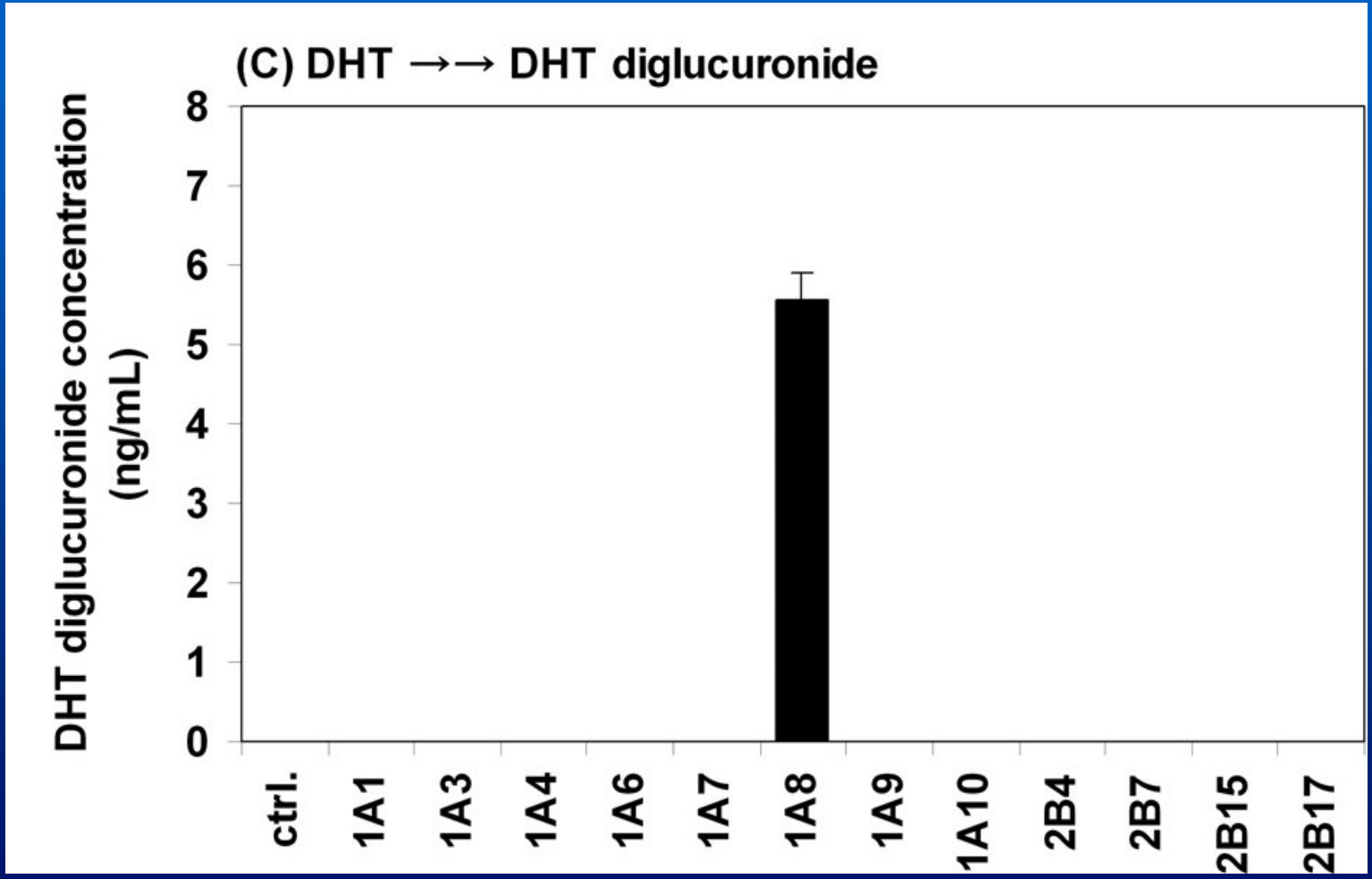




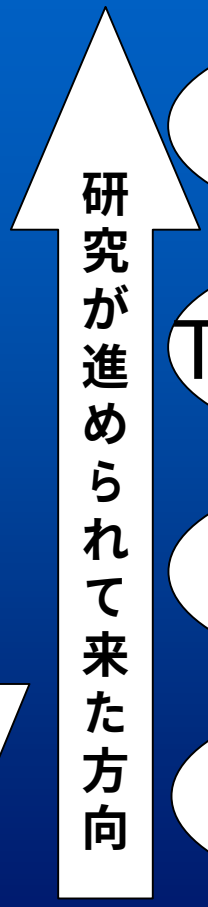
Glucuronidation of DHT and DHT monoglucuronide by recombinant human UGTs



Glucuronidation of DHT to Diglucuronide by recombinant human UGTs



低分子の動きから高分子の動きへ



Genome

Transcriptome

Proteome

Metabolome

Molecular biologist → **DNA、RNA**

Biochemists

Biochemists → **酵素、蛋白質**

MS scientists
Analytical chemists → **尿、血液、胆汁**
Organic chemists

Acknowledgments

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山田真起子　堀口正明　佐復直純　高仲 郁
河合賢司　清水和巳　寺尾俊夫　佐々木幸雄
廣田孝司　椿 秀美　栗原厚　吉ヶ江泰志
数井美穂　萩原克宣　本多伴世

熊本大学大学院薬学研究科
小田切優樹