REVIEW

Organic Stereochemistry

Part 7[1])

The Concept of Substrate Stereoselectivity in Biochemistry and Xenobiotic Metabolism

by **Bernard Testa**

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This review continues a general presentation of the principles of stereochemistry with special emphasis on the biomedicinal sciences. Here, we discuss and illustrate the phenomenon of substrate stereoselectivity in biochemistry (endogenous metabolism) and principally in xenobiochemistry or drug metabolism.
The review begins with an overview of the stereoselective processes occurring in the biomedicinal sciences. The general rule is for distinct stereoisomers, be they enantiomers or diastereoisomers, to elicit different pharmacological responses (*Part 5*), to a lesser extent be transported with different efficacies (*Part 5*), and to be metabolized at different rates (this *Part*). In other words, biological environments discriminate between stereoisomers both when acting on them and when being acted upon by them. The concept of substrate stereoselectivity describes this phenomenon in endogenous biochemistry and xenobiotic metabolism, as discussed and illustrated in the present *Part*. The sister concept of product stereoselectivity will be presented in *Part 8*.

This seventh review continues our series on Organic Stereochemistry by focusing on a major concept in biochemistry and xenobiotic metabolism, namely that of *substrate stereoselectivity*. This concept describes the well-known fact that stereoisomers are generally metabolized at different rates. Also implicit in this definition is the fact that their metabolic patterns may differ, all experimental conditions being equal. As we shall see, this concept alone does not cover the entire field of endogenous and exogenous metabolism, since it neglects two other very important and related concepts, namely *prostereoisomerism* and *product stereoselectivity* which will be covered in *Part 8*.

The discrimination between substrate and product stereoselectivities owes much to the pioneering work of *Vladimir Prelog*, *Nobel* Laureate in 1975 and a founding father of modern stereochemistry. Beginning in the mid-fifties and for many years thereafter, he investigated the stereoselective reduction of xenobiotic ketones by microorganisms, leading him to *conceptualize* a clear discrimination between *a*) the differential metabolism of two stereoisomeric substrates (substrate stereoselectivity), and *b*) the differential formation of two stereoisomeric metabolites produced by the creation of a stereogenic element (often a center of chirality) in a single substrate (product stereoselectivity; see, *e.g.*, [1][2]). The layout of *Parts 7* and *8* follows *Prelog*'s lead.

[1]) For the other *Parts*, see *Helv. Chim. Acta* **2013**, *96*, 1 – 3.

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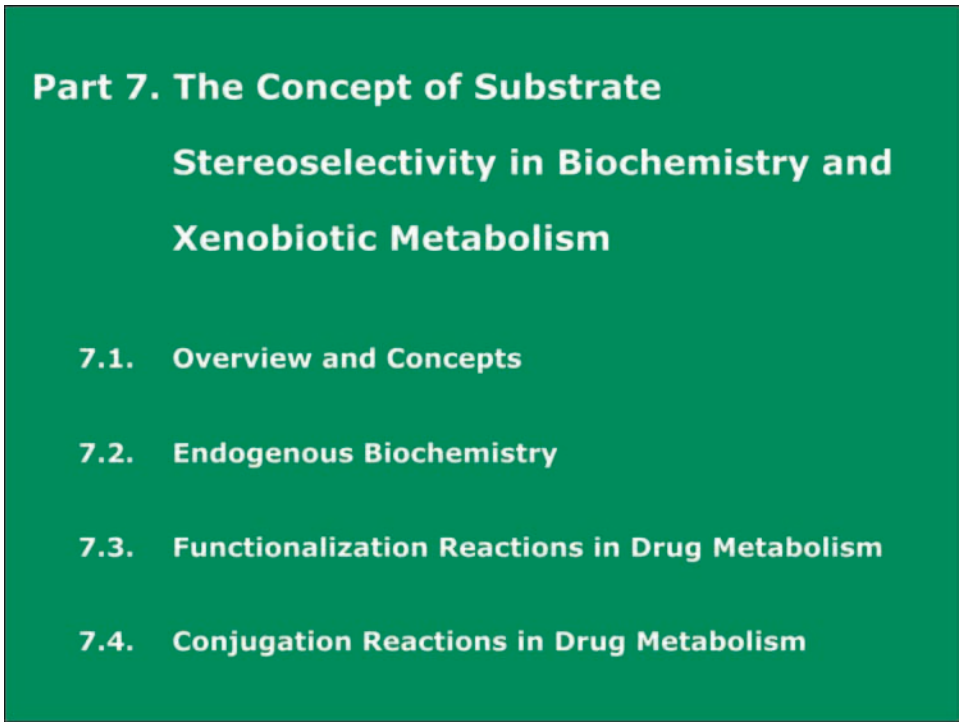


Fig. 7.1. The *content of this Part* is summarized here and quite logically begins with a clarification of concepts. There is indeed a confusing lack of clarity in the literature due to ambiguous terms and poorly defined concepts. Our exposition of stereoselectivity in the biochemistry of endogenous and exogenous (xenobiotic) compounds is based on the key distinction between *substrate* and *product stereoselectivities*, to be presented in this and the following *Part*, respectively.

Following an overview of concepts, three sections will serve to illustrate *substrate stereoselectivity* by presenting a variety of relevant examples. First, we shall take a look at *endogenous metabolism*, which involves the anabolism (synthesis) and catabolism (degradation) of endogenous compounds. This will be followed by two sections on substrate stereoselectivity in the metabolism of drugs and other xenobiotics, one covering *functionalizations (hydrolysis and redox)* [3], the other *conjugations* [4].

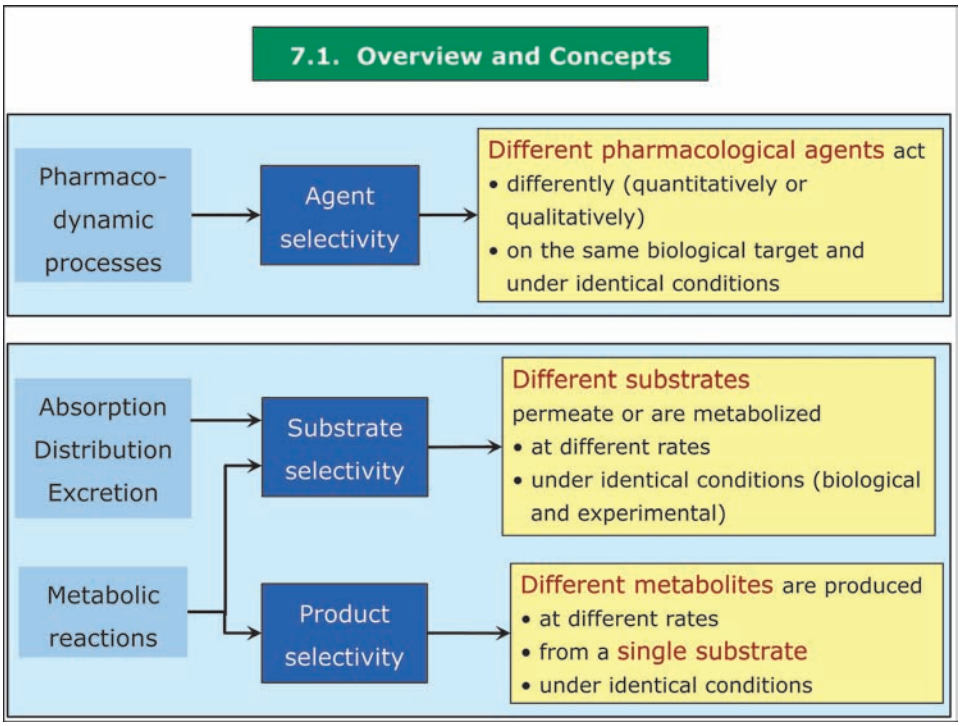


Fig. 7.2. In *Part 5*, we discussed *pharmacodynamic events* and saw how stereoisomeric drugs or toxins interact differently with a given molecular site of action (a '*target*') and thereby elicit a stereoselective response. In shorthand, what we discussed was '*agent stereoselectivity*', a specific case within the broader concept of *agent selectivity* as defined here. In addition, in *Part 5* we also considered *pharmacokinetic events* that leave the substrate unchanged, namely *absorption*, *distribution*, and *excretion*, be they *passive* (*i.e.*, occurring spontaneously down a concentration gradient) or *active* (*i.e.*, mediated by energy-consuming transporters, generally against a gradient). The label of *substrate stereoselectivity*, a particular case of *substrate selectivity*, applies here.

When it comes to *metabolism* (*i.e.*, biotransformation), things get more complex, as this *Figure* summarizes. Here, indeed two very distinct situations may occur – even simultaneously. First, the concept of *substrate selectivity* also applies to metabolism, meaning that chemically distinct or isomeric substrates are metabolized at different rates and/or present different metabolic patterns. But, as mentioned above, it was the merit of *Prelog* [1][2] to clearly conceptualize a situation unique to biotransformation, namely the fact he abundantly exemplified of a *new center of chirality* being metabolically created in a substrate molecule, thereby producing two stereoisomeric metabolites in different proportions. This phenomenon comes under the name of *product stereoselectivity*, a particular case of *product selectivity*.

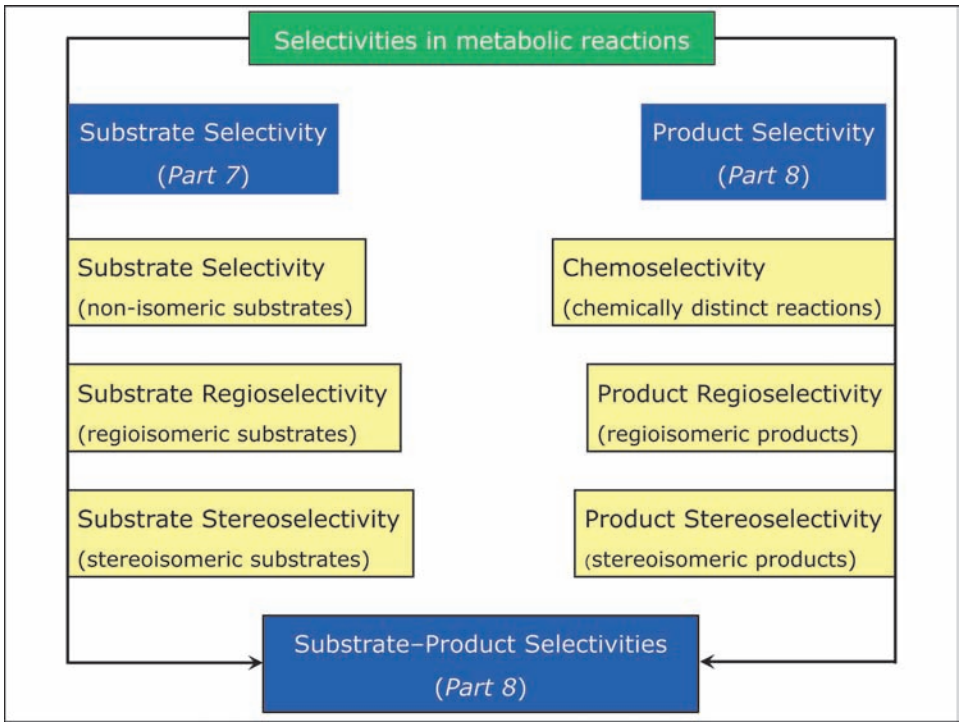


Fig. 7.3. Stereoselective processes in drug metabolism have been studied systematically for the last six decades [5], but patterns were difficult to discern among the many examples uncovered. As mentioned above, it was the great contribution of *Prelog*, on the basis of his results with xenobiotic metabolism in microorganisms (see, *e.g.*, [1]), to propose a clarification based on the concepts of substrate and product stereoselectivities [6–20]. These concepts proved fertile enough to be expanded by including other aspects of isomerism (see *Part 1*) and even non-isomeric substrates and metabolites [21][22].

A *structural subclassification* of substrate and product selectivities is proposed here with the understanding that *identical biological and experimental conditions* are assumed throughout. The presentation of *stereoselectivities* is what occupies us here, but the *Figure* also mentions *regioselectivities*, *i.e.*, regioisomeric substrates or products [21]. *Substrate selectivity in a broad sense* covers different, non-isomeric compounds and is straightforward. The same cannot be said of *chemoselectivity*, given that some enzymes are capable of chemically distinct reactions, *e.g.*, *C*- and *N*-oxygenations by cytochromes P450, or *O*- and *N*-glucuronidations by glucuronosyltransferases [3][4]. And, as will be discussed in *Part 8*, there are composite cases in which product stereoselectivity differs from one stereoisomeric substrate to the other (*substrate–product stereoselectivity*).

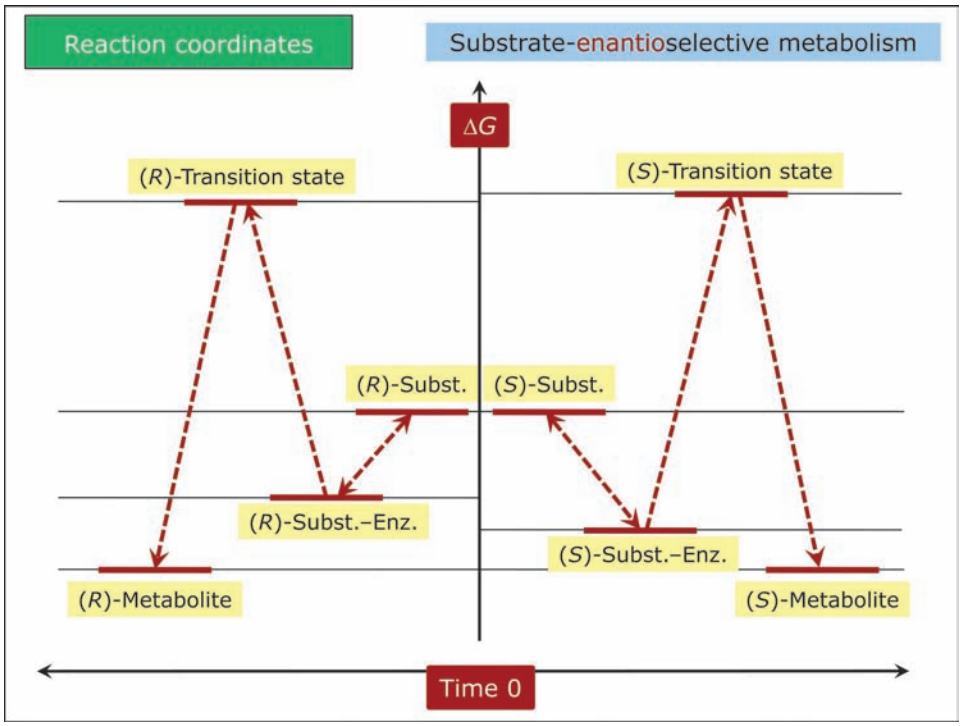


Fig. 7.4 and 7.5. The *contribution of binding* to *substrate enantioselectivity*, namely the differental binding of enantiomers to an enzyme, is due to the chiral nature of the binding site, as schematized in the *attachment* model of *Easson* and *Stedman* [23] (*Part 5*). But does this model account fully for substrate enantioselectivity? As with chiral recognition at pharmacological receptors (*Part 5*), stereoselectivity can also occur at the activation step, which in biochemistry is called the *catalytic step*.

This is illustrated here with a *thermodynamic argument*, namely by plotting the *reaction coordinates* of the two enantiomeric substrates labeled as *(*R*)-Subst.* and *(*S*)-Subst.* [24]. These, by definition, have identical internal energies (plotted as *Gibbs energy* = 'free energy' = ΔG). Each enantiomer binds reversibly to the (chiral) enzymatic site, giving two *substrate–enzyme complexes* (labeled *Subst.-Enz.*) which are diastereoisomeric and thus differ in their energies. This implies a *differential affinity* to the enzyme, the magnitude of which will depend on the different energies of the two complexes. In our quite arbitrary example, the (*S*)-enantiomer forms a more stable complex. In enzyme kinetics experiments, *binding affinity* is usually determined as the *Michaelis–Menten constant* K_M (in molar concentration of substrate).

The *catalytic step* of the two enantiomers then passes through a *transition state.* The energy difference between these two transition states is expected to be modest, as it depends essentially on the activation energy of the same reaction occurring in the two enantiomers. Here, the (*R*)-substrate is arbitrarily chosen to react faster. Assuming that the substrates neither lose their stereogenic center nor acquire a new one during the reaction (the latter being a case of substrate–product stereoselectivity; *Part 8*), the

*(*R*)-Metabolite* and the *(*S*)-Metabolite* will be enantiomers. The experimental parameters that best approximate this catalytic step are the *maximal rate at saturation* (V_{max} in molar concentration of substrate per time per molar concentration of catalyst) or the *turnover number* k_{cat} (in 1/time).

In summary, substrate enantioselectivity as schematized here results from a balance between two differences in free energy, first between the two enzyme–substrate complexes and second, between the two transition states. This balance is represented by the *catalytic efficiency*, *i.e.*, V_{max}/K_M or k_{cat}/K_M

A comparable argument is applicable to the case of *substrate diastereoselectivity* (*Fig. 7.5*). The two diastereoisomers differ in their internal energy, the *(*E*)-Subst.* being arbitrarily chosen as more stable than *(*Z*)-Subst*. The diastereoisomeric *enzyme–substrate complexes* and *transition states* again differ in their stability. But the major difference with substrate enantioselectivity is that the two metabolites, provided they have not lost their stereogenic element during the biotransformation reaction, may show a differential stability paralleling that of the substrates.

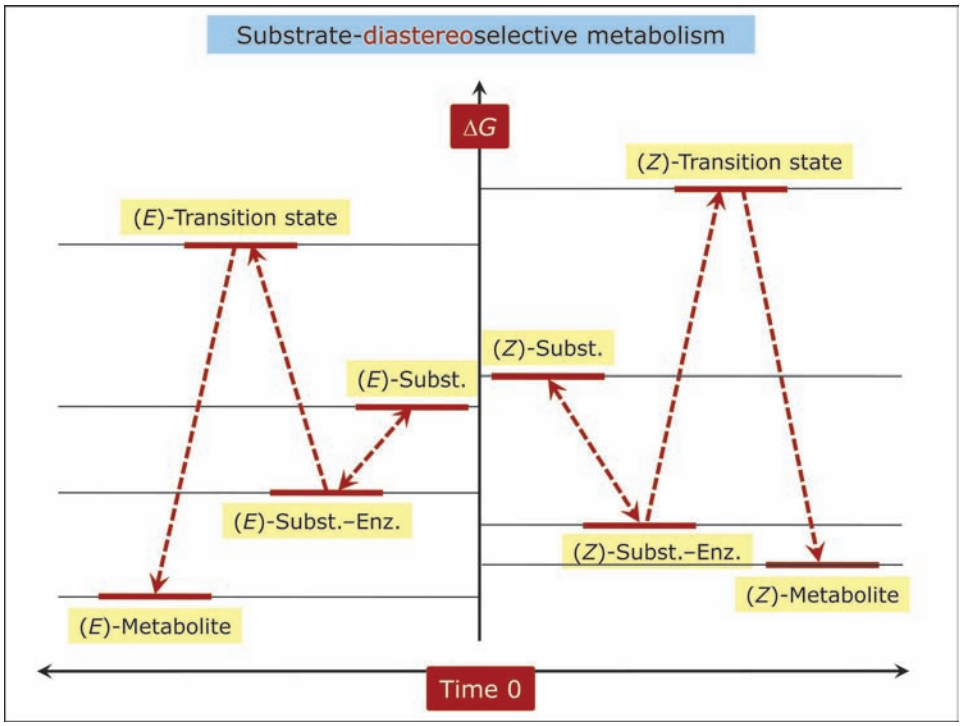


Fig. 7.5.

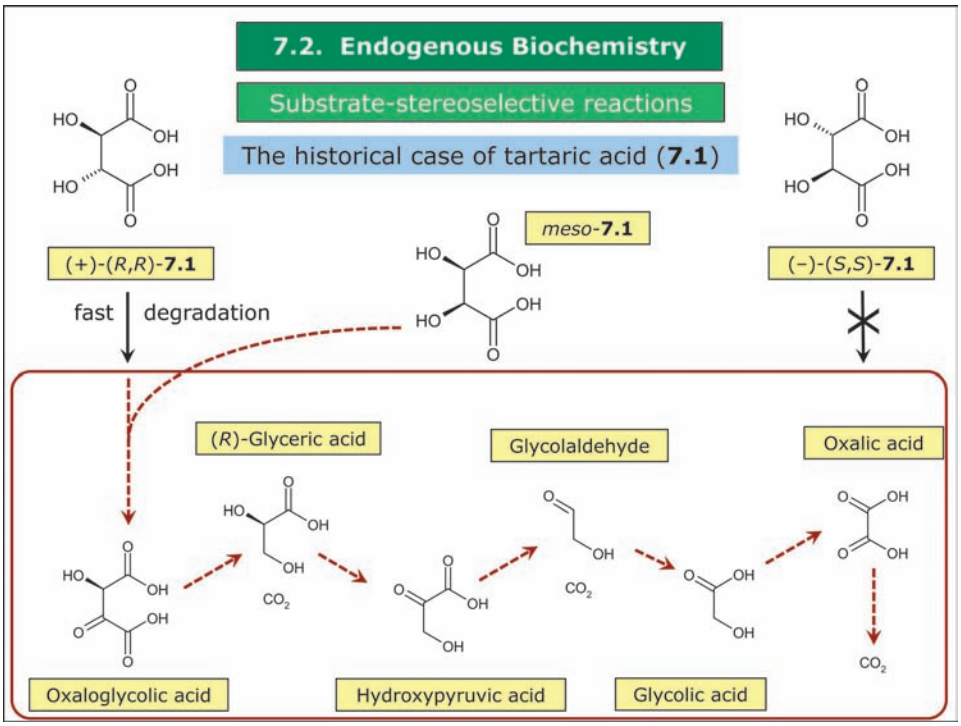


Fig. 7.6. We now turn our attention to *stereoselectivity in endogenous biochemistry*, that is in the anabolism and catabolism of endogenous compounds such as fatty acids, amino acids, hormones, and secondary metabolites [25–28]. For example, it is a well-known fact that proteinogenic *amino acids have the* L*-configuration*, which in all cases except cysteine translates as (*S*). This is not to say that D-amino acids do not exist, simply that they are rare and their physiological roles are limited and poorly understood yet real [29]. Substrate and product enantiospecific reactions are obviously needed to achieve such exquisite stereospecificity.

In this *Figure*, we summarize the *first discovery of substrate enantioselectivity*, as reported by *Louis Pasteur* in 1857 and 1858, and cogently discussed by *Gal* [30]. Several years earlier, *Pasteur* had achieved the *first physical separation of enantiomers*, that of (+)- and (−)-tartaric acid (**7.1**), by hand-picking their enantiomorphous crystals and measuring the optical rotation of their solutions. In further studies, he incubated racemic ammonium tartrate with *microorganisms* (most likely yeast) and found that the (+)-form was consumed rapidly, whereas the (−)-form was not, and that a gas was released, most likely CO_2. The two reactions investigated by *Pasteur* are represented by black arrows, while the dotted red arrows represent more recent knowledge; thus, *meso*-tartrate was discovered by *Pasteur* years later, and the red box contains one of the conceivable breakdown routes of tartaric acid.

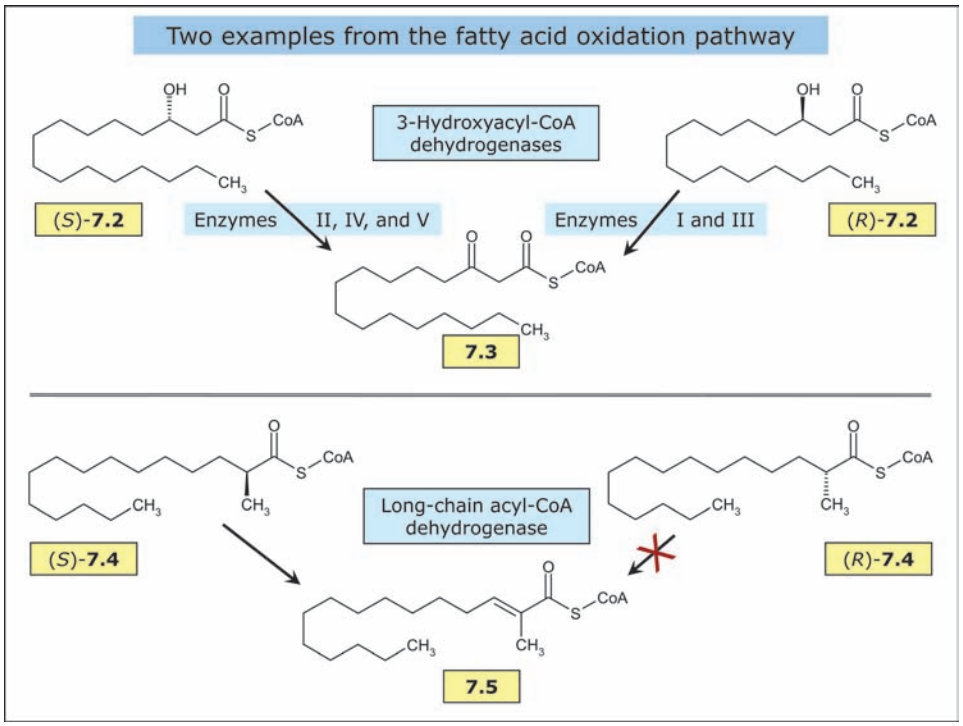


Fig. 7.7. The *Figure* presents two examples from the field of *fatty acid oxidation*. Taking palmitic acid as an example of '*normal' (straight-chain) fatty acids*, its first cycle of *β-oxidation* involves *a*) conjugation with coenzyme A (CoA), *b*) 2,3-desaturation to the Δ^2-enoyl metabolite, *c*) hydration to *3-hydroxypalmitoyl-CoA* (**7.2**), *d*) dehydrogenation to *3-oxopalmitoyl-CoA* (**7.3**), and *e*) C_2-shortening by loss of acetyl-CoA. Significantly, the hydration step *c* produces *(*S*)-3-hydroxyacyl-CoA* and is thus *product-stereoselective* (*Part 8*). The upper part of the *Figure* shows step *d*, the product of which has *lost the stereogenic center* in the acyl moiety.

β-Oxidation occurs in mitochondria and/or in peroxisomes. Given the (*S*)-configuration of 3-hydroxyacyl-CoA, the dehydrogenation step *d* is *substrate-enantioselective* as expected. However, this is not an absolute rule, and some multifunctional proteins (MFP) involved in *β*-oxidation have been found to show the opposite enantioselectivity when forming *3-oxopalmitoyl-CoA* (**7.3**). Thus, five *3-hydroxyacyl-CoA dehydrogenases* (labeled I to V) were isolated from rat liver peroxisomes [31]; three were specific for the (*S*)-form, and two for the (*R*)-form. This suggests an evolutionary benefit due to the recuperation of the 'wrongly' configured (*R*)-3-hydroxyacyl-CoA conjugates.

A different story is told by *2-methyl-branched fatty acids* (lower part of the *Figure*), some of which are found in humans and animals, *e.g.*, (2*S*)- and (2*R*)-pristanic acid. Like *n*-fatty acids, they are conjugated to the *(2*S*)- and (2*R*)-pristanoyl-CoA epimers* [4][32]. The former epimer, but not the latter, is a substrate for *β*-oxidation due to the substrate selectivity of *long-chain acyl-CoA dehydrogenase* which catalyzes the 2,3-

desaturation step. With *(*S*)- and (*R*)-2-methylpentadecanoyl-CoA* (**7.4**) as model substrates, it was shown that only the former yielded the trans-Δ^2-*ene-2-methyl-acyl-CoA* metabolite **7.5** capable of undergoing β-oxidation [33]. Note that the (*R*)-2-methylacyl-CoA enantiomer does not accumulate in the body but is a substrate of 2-methylacyl-CoA 2-epimerase which catalyzes its inversion of configuration to the metabolically labile (*S*)-enantiomer, thus avoiding accumulation of (2*R*)-pristanic acid [34–36].

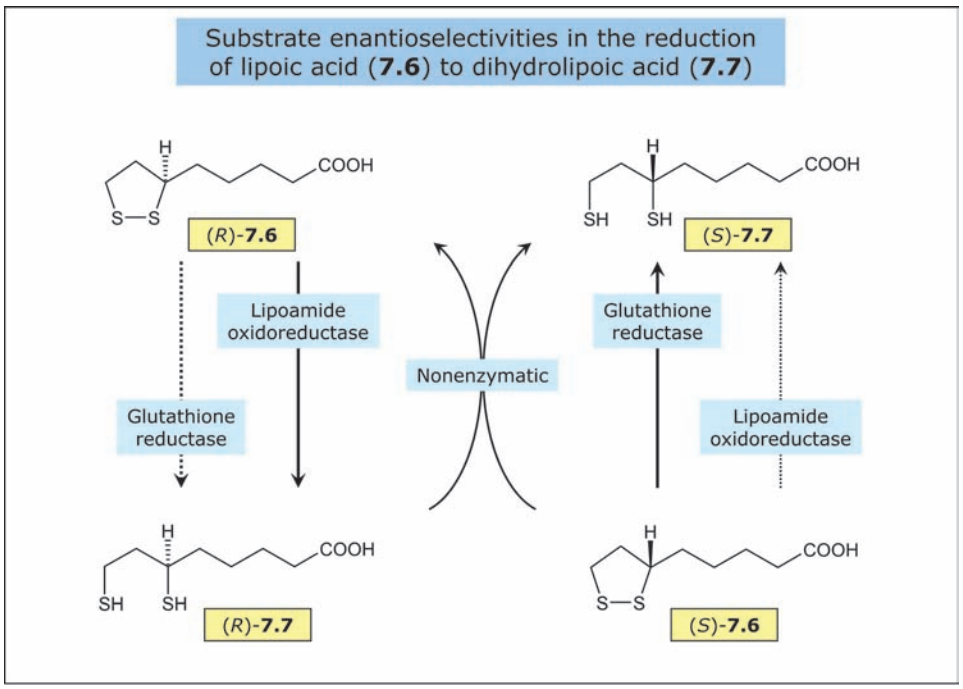


Fig. 7.8. The example of *lipoic acid* (**7.6**) was selected for two reasons, because it is both an endogenous and an exogenous compound, and to illustrate some intricacies found in stereoselective bioreactions. The compound, also known as α-lipoic acid, can be considered as a S-containing derivative of octanoic acid. Its (*R*)-enantiomer is the natural form and serves as an essential cofactor in some mitochondrial enzyme complexes where it is found as protein-bound lipoamide. But lipoic acid is also used as a *nutritional supplement*, mainly as the racemate but sometimes as the (*R*)-form. Its claimed *in vivo* antioxidant properties reside in the two SH groups of dihydrolipoic acid (**7.7**), and it is, therefore, of interest to understand the characteristics of its bioreduction [37][38].

The main enzyme involved in lipoic acid reduction was found to be *lipoamide oxidoreductase*, also known as dihydrolipoamide dehydrogenase. This enzyme shows high enantioselectivity, its activity being much higher toward (*R*)- than (*S*)-lipoic acid. Glutathione reductase, whose overall reductive activity toward lipoic acid is low

compared to lipoamide oxidoreductase, displayed the opposite enantioselectivity, with a moderate preference for the (*S*)-enantiomer. But the story does not end here, since it was found that (*R*)-dihydrolipoic acid and other 1,3-dithiols mediate the *nonenzymatic reduction of* (S)*-lipoic acid*. The reverse reaction *i.e.*, nonenzymatic reduction of (*R*)-lipoic acid by (*S*)-dihydrolipoic acid, also occurs but is not shown here.

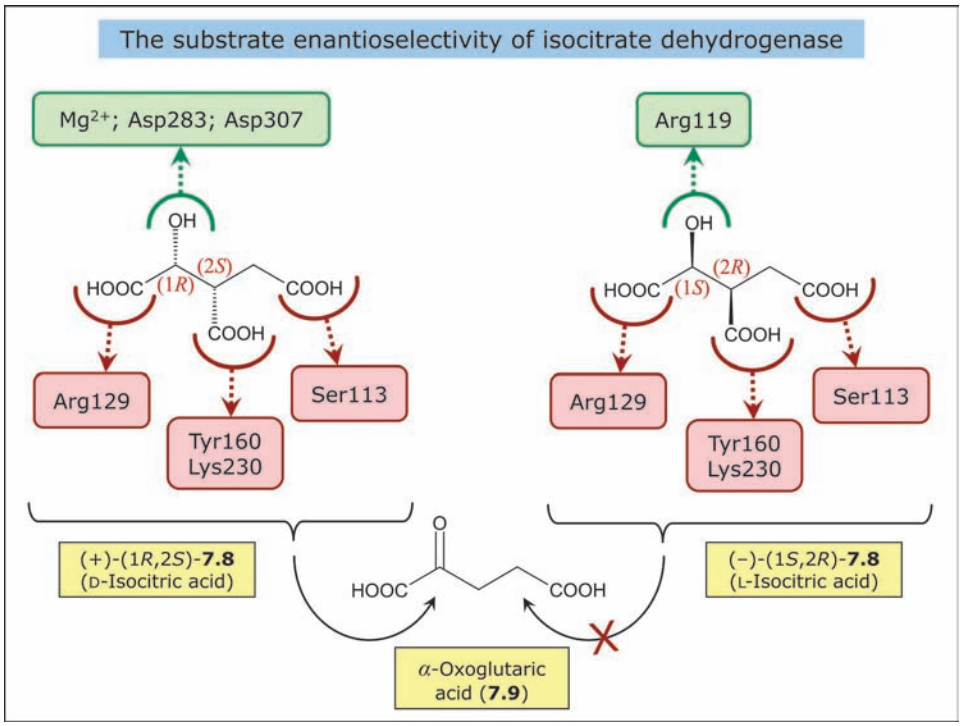


Fig. 7.9. In *Part 5*, we met the four-location model of chiral recognition proposed by *Mesecar* and *Koshland* who deduced it from a crystallographic study of *isocitrate dehydrogenase* [39]. The substrate molecule, *isocitric acid* (**7.8**), contains three COOH groups whose sites of binding are the same for both the (+)-(1*R*,2*S*)- and (−)-(1*S*,2*R*)-enantiomers, namely Arg129, Tyr160 with Lys230, and Ser113. As a result, these three sites alone would fail to elicit any enantioselectivity. A fourth group is thus necessary in the enzymatic site to allow enantioselective recognition. The OH group in isocitric acid targets this fourth group, which proved to be *Arg119* in the metal-free, non-functional enzyme, and Mg^{2+} in the *Mg-containing functional enzyme*. Only the non-substrate (−)-(1*S*,2*R*)-isocitric acid (L-isocitric acid) was found to bind to the metal-free enzyme, whereas only the *physiological substrate* (1*R*,2*S*)-(+)-isocitric acid (D-isocitric acid) was bound to the Mg^{2+}-containing enzyme. In other words, enantioselectivity was not seen in the binding step, since the two enantiomers of isocitric acid were bound to the enzyme, but at the catalytic step. The product of the enzymatic reaction is *α-oxoglutaric acid* (**7.9**) formed by decarboxylation at C(3) and dehydrogenation of the 2-OH group.

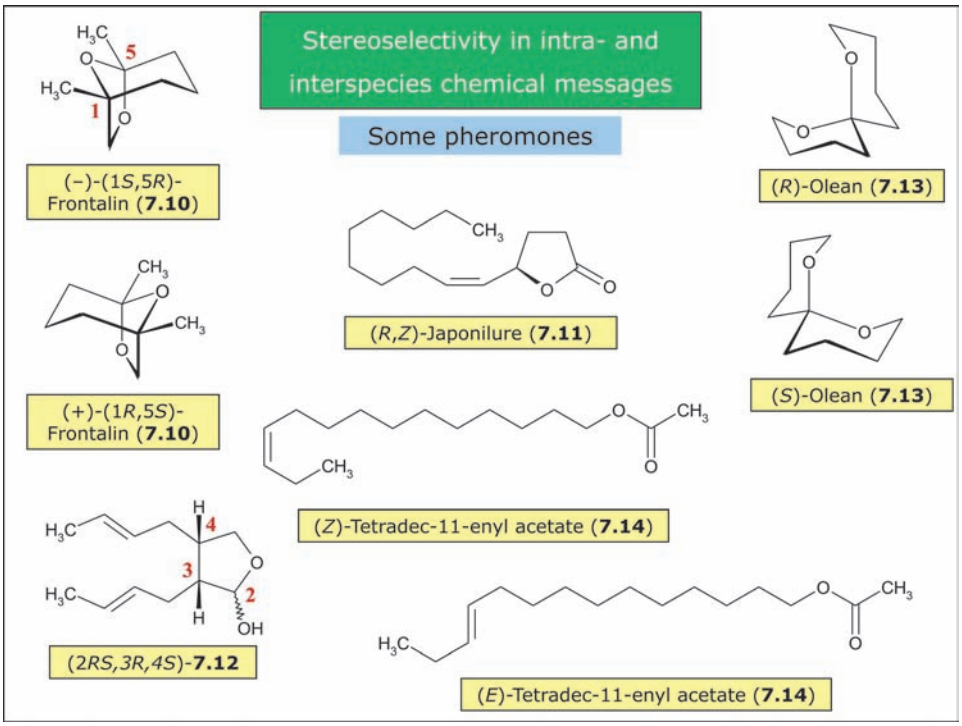


Fig. 7.10. The above examples illustrate the significance of substrate-stereoselective reactions in the anabolism and catabolism of endogenous compounds. While a discussion on macroscopic aspects of biochirality [40][41] is outside the scope of this work, we wish to draw the reader's attention to the significance of *stereoselectivity in the chemical messages* exchanged between individuals of the same or different species. The stereoisomeric composition of these compounds is often a critical determinant in their effects, as illustrated below, implying that their stereoselective biosynthesis was fine-tuned by evolution.

Pheromones are chemical signals emitted by individuals to trigger a social response in other members of the same species. Some allow food trails to be traced, others are alarm or sex signals, or they affect various aspects of behavior or physiology [42]. Most investigations have focused on insect pheromones, but some plants and vertebrates also use pheromones. In this *Figure*, we present a few examples of animal pheromones [43–45]. *Frontalin* (**7.10**) is an aggregation pheromone of the western pine beetle and other species of bark beetles; only the (−)-(1*S*,5*R*)-enantiomer was active, at least in the former species. Frontalin thus illustrates the case where *one stereoisomer is active and the other not*. A different situation occurs when one stereoisomer is active and the other an *inhibitor*, rendering the racemate inactive. This was observed for example with *japonilure* (**7.11**), the female-produced sex pheromone of the Japanese beetle; here, the active enantiomer is the *(*R,Z*)-form*, while the (*S*,*Z*)-enantiomer strongly inhibited the action of the (*R*,*Z*)-form. Although the (*E*)-isomers and the saturated analog of

japonilure were present in the material obtained from females, their role in mediating the insect's behavior was unclear [46]. The careful reader will have noted that **7.11** contains *two stereogenic elements*, a stereogenic center and an element of (*E*,*Z*)-diastereoisomerism. This, however, does not prevent the (*R*,*Z*)- and (*S*,*Z*)-forms to be non-superimposable mirror images, *i.e.*, enantiomers, as indeed stated in the original reference [46].

The *male spined citrus bug pheromone* **7.12** contains a configurationally labile hemiacetal center C(2). It is produced naturally as an active mixture of the synthetic (2*RS*,3*R*,4*S*)-epimers, but it is interesting to note that the synthetic (2*RS*,3*S*,4*R*)-pair of epimers is also active. In other words, what we have here is a case where *both the natural and the synthetic stereoisomer are active*. An unusual case is offered by *olean* (**7.13**), the female-produced sex pheromone of the olive fruit fly. When prepared and field-tested, (*R*)- and (*S*)-oleans revealed that the *(*R*)-enantiomer acted on males* and the *(*S*)-enantiomer on females* [43–45]. The natural pheromone was found to be the racemate, meaning that an emitting female would act both on males and on herself and other females.

Even more astonishing is the activity of the two geometrical isomers of *tetradec-11-enyl acetate* (**7.14**), the sex pheromone of agriculturally important insect pests, namely the European corn borer and other moths [47][48]. Corn borer females use this pheromone to attract males. Studies in Iowa State using the pure (*Z*)-isomer revealed a weak activity, while the (*E*)-isomer was inactive. Mixtures of the two diastereoisomers in different proportions elicited a response which was maximal for an *(*E*)/(*Z*)-mixture in a 3:97 ratio*. In contrast, studies in New York State provided an optimal *ratio of 98:2*. The difference between the insects preferring the (*E*)-isomer (*the* E-*race*) and those preferring the (*Z*)-isomer (*the* Z-*race*) arises from a genetic polymorphism of the fatty-acyl reductases which reduce either (*E*)- or (*Z*)-tetradec-11-enoic acid with very high substrate stereoselectivity to (*E*)- or (*Z*)-tetradec-11-en-1-ol. As a result, the *E*- and *Z*-races of corn borers were postulated to be *en route* to species divergence (incipient species) [45].

Returning to *frontalin* (**7.10**), its relevance extends well beyond the insect world as it is also a pheromone in *elephants* [49]. Male elephants experience an annual period of heightened sexual activity and aggressivity known as '*musth*'. Both enantiomers of frontalin have been shown to be emitted by male Asian elephants in a stereoisomeric ratio that changed with the animal's age and stage of musth. Frontalin secretion became detectable at ages 13–20 years and rose *ca.* 15-fold over a 25-year period, while the enantiomeric ratio evolved from an average (+)/(−) ratio of *ca.* 60:40 to near racemic. There were also large fluctuations in enantiomeric ratios as musth progressed, but mid-musth was generally characterized by a near racemic ratio. Importantly, male and female elephants perceived these differences in amounts and enantiomeric ratios, and reacted accordingly. In particular, high emission of racemic frontalin repulsed males, and luteal-phase and pregnant females, but they attracted follicular-phase females.

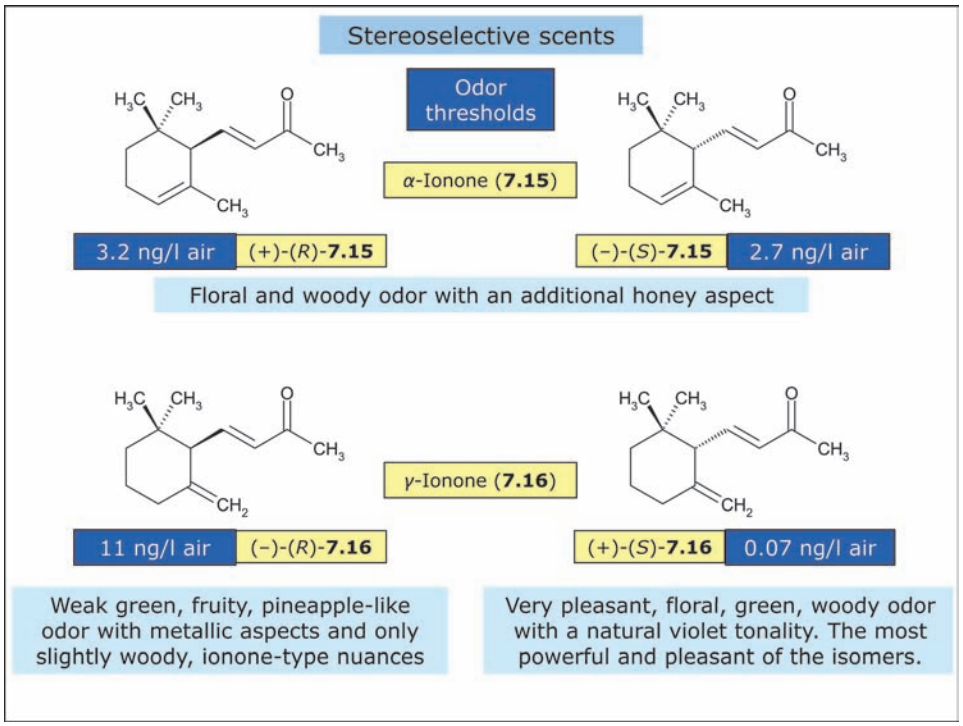


Fig. 7.11. To conclude this *Section* with a flowery note, we highlight the *stereoselectivity of fragrances* as agonists of the human olfactory receptors [50–56]. As such, this topic is as close to molecular pharmacology as it is to biochemistry. Many recent fragrances are synthetic perfume components, but here we are interested in natural volatiles produced by flowers to attract their pollinators. In other words, these are not (intraspecies) pheromones, but *interspecies signals* which have evolved to a high level of structural complexity, including stereochemical features.

Ionones are produced by violet flowers and contribute significantly to their refined odor. Among its various analogs, regioisomers and stereoisomers, particular attention has been given to *α-ionone* (**7.15**) and *γ-ionone* (**7.16**) [57][58]. Both compounds are chiral, their enantiomers being shown in the *Figure* together with their *odor thresholds* (*i.e.*, the minimal air concentration detectable by human subjects) and their *odor profile* as defined by experts (the so-called 'noses'). There is clearly little difference in the human perception of the two α-ionones (**7.15**), their profile and threshold being similar within experimental errors. In contrast, a large difference is detected between the enantiomers of γ-ionone (**7.16**), (+)-(*S*)-γ-ionone being by far the most active on the human olfactory system and eliciting the most pleasant effect.

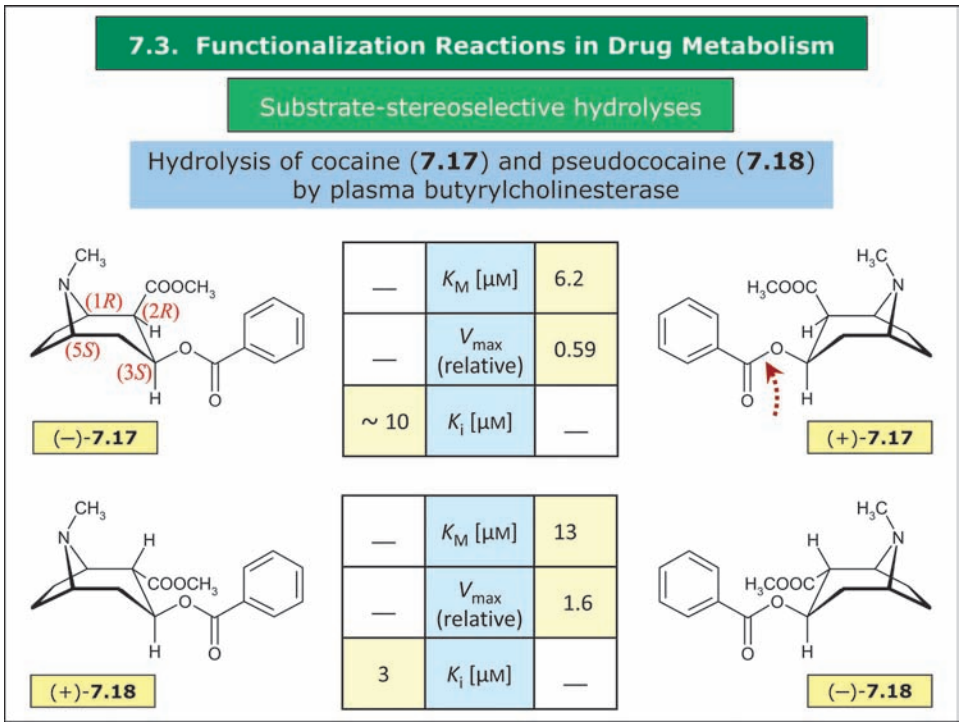


Fig. 7.12. We now move to the biochemical reactions that metabolize *xenobiotics* (*i.e.*, drugs and other 'foreign' compounds) [3][4][59]. Reactions of *functionalization*, namely the creation or transformation of a functional group in a substrate, will be exemplified first, and reactions of *conjugation* will follow. To begin with reactions of *hydrolysis* [3][60], a rather straightforward example is provided by *cocaine* (**7.17**) and *pseudococaine* (**7.18**). The structure of cocaine covers eight stereoisomers [61], four of which ((+)- and (−)-cocaine and (+)- and (−)-pseudococaine) were investigated for their hydrolyses by *plasma butyrylcholinesterase* (BChE; EC 3.1.1.8) [62]. This enzyme is regioselective in that it cleaves cocaine at the benzoyloxy bridge, as opposed to carboxylesterases (CES; EC 3.1.1.1) which can cleave both ester bridges [3][60]. As seen, the *natural and highly neuroactive (−)-cocaine* is highly resistant to BChE-catalyzed hydrolysis and is, in fact, an inhibitor of the enzyme, as assessed by its K_i value. As a result, (−)-cocaine is metabolized mainly in the liver but not in the blood circulation, a pharmacokinetic factor that plays an obvious role in its duration of action. The resistance of (−)-cocaine to BChE hydrolysis is most likely due to a binding mode that does not allow the target ester bridge to make contact with the catalytic triad [3][60]. The same behavior is detected with *(+)-pseudococaine*. In contrast, *(+)-cocaine* and *(−)-pseudococaine* are good substrates of BChE; this is due to differences in both *binding affinity* (as expressed by the *Michaelis–Menten* constant, K_M) and the *maximal rate at saturation* (expressed by V_{max}), while the *catalytic efficiencies* (V_{max}/K_M) are comparable.

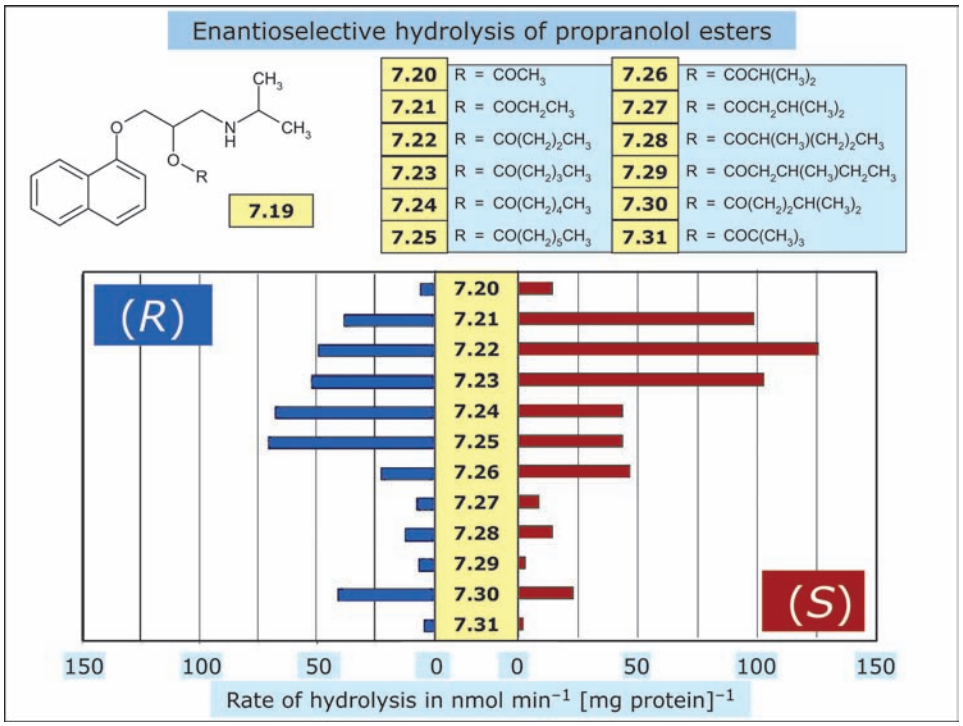


Fig. 7.13. Metabolic studies using chiral series of homologous and analogous substrates may also lead to useful conclusions, one of which being that even small differences in structure may lead to marked differences in enantioselectivity. This is exemplified here with a series of linear and branched acyl esters of *propranolol* (**7.19**, R = H), the archetypal β-blocker [63][64]. Like all other drugs in this class, this compound is chiral, and there have been attempts to improve its modest bioavailability by decreasing its first-pass metabolism using a *prodrug strategy*. The ester prodrugs shown here are n-*acyl esters*, **7.20 – 7.25**, and *branched-acyl esters*, **7.26 – 7.31**. Their rates of hydrolysis was investigated *in vitro* by incubating the racemates and monitoring the metabolite (*i.e.*, propranolol) by chiral HPLC. The results shown were obtained with rat liver microsomes (a biological preparation rich in *carboxylesterases*). For the propanoate to heptanoate **7.21** – **7.25**, respectively, hydrolysis was fast and *favored the (*S*)-esters* only up to the pentanoate **7.23**. For the two higher homologs, hexanoate and heptanoate **7.24** and **7.25**, respectively, hydrolysis *favored the (*R*)-esters*. For the *branched acyl analogs*, hydrolysis and stereoselectivity were low in some cases (for **7.27**, **7.28**, **7.29**, and **7.31**); hydrolysis was faster and of opposite stereoselectivity for the isobutanoate and isohexanoate **7.26** and **7.30**, respectively. In other words, no clear trend emerged from the branched-alkyl series. Different results were obtained with rat plasma (which contains both BChE and CES), where in most cases the (*R*)-esters were hydrolyzed faster.

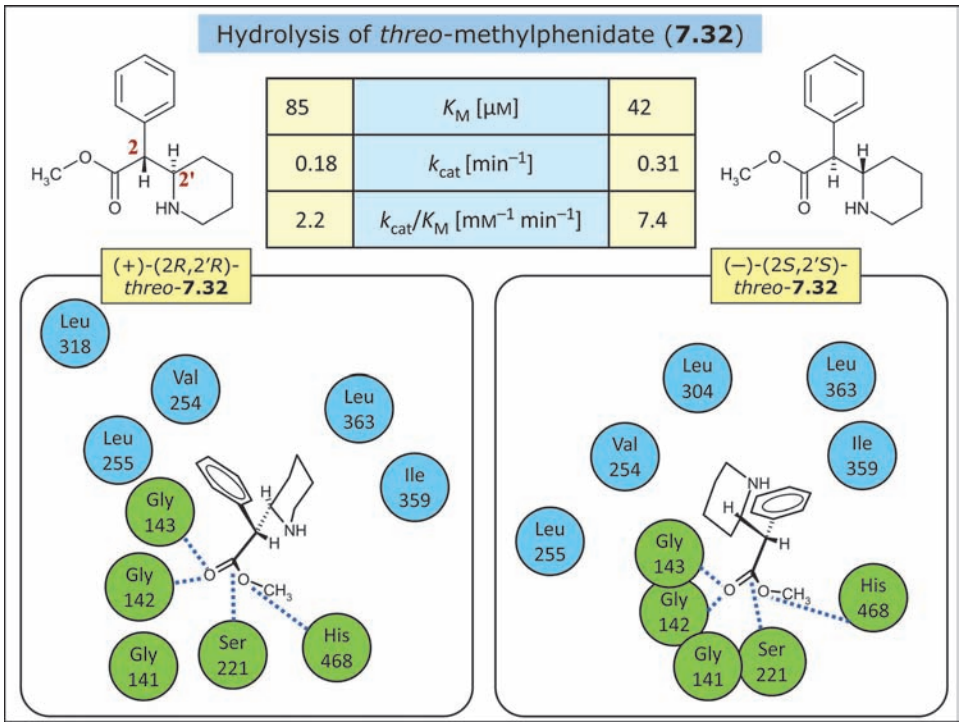
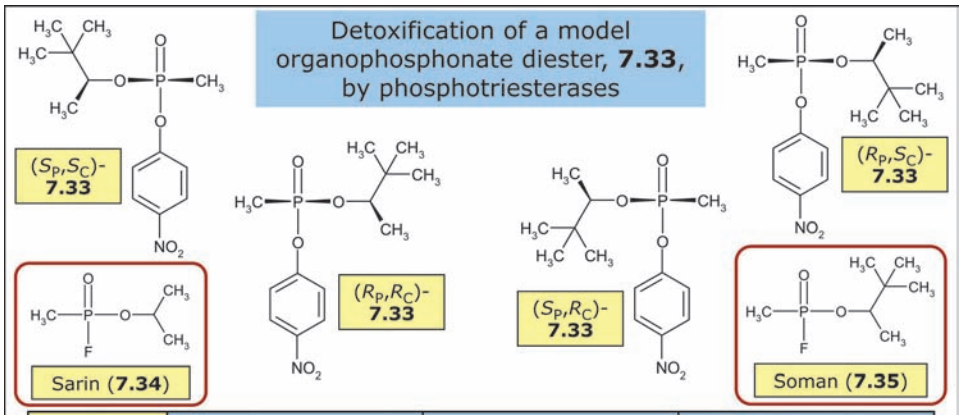


Fig. 7.14. Molecular modeling can help us understand stereoselectivity at the molecular level, and some papers have addressed this for carboxylesterase-catalyzed hydrolysis [65–67].

Methylphenidate is a case in point. This psychostimulant drug is used as the racemic *threo*-pair **7.32**, the (+)-(2*R*,2′*R*)-enantiomer being the pharmacologically active one [68][69]. There is also a marked metabolic difference between the two *threo*-enantiomers in that the levorotatory form undergoes faster first-pass elimination *in vivo* and is hydrolyzed faster *in vitro*. The results shown here were obtained with the separate enantiomers using purified native human carboxylesterase 1 (CES1) [67]. The measured *catalytic efficiencies* (k_{cat}/K_M) confirm that the (−)-(2*S*,2′*S*)-form is a three- to fourfold better substrate than its enantiomer. In more details, the *binding affinity* (K_M) of the *levo*-form is about twice that of the *dextro*-form, while its *turnover number* (k_{cat}) is about double.

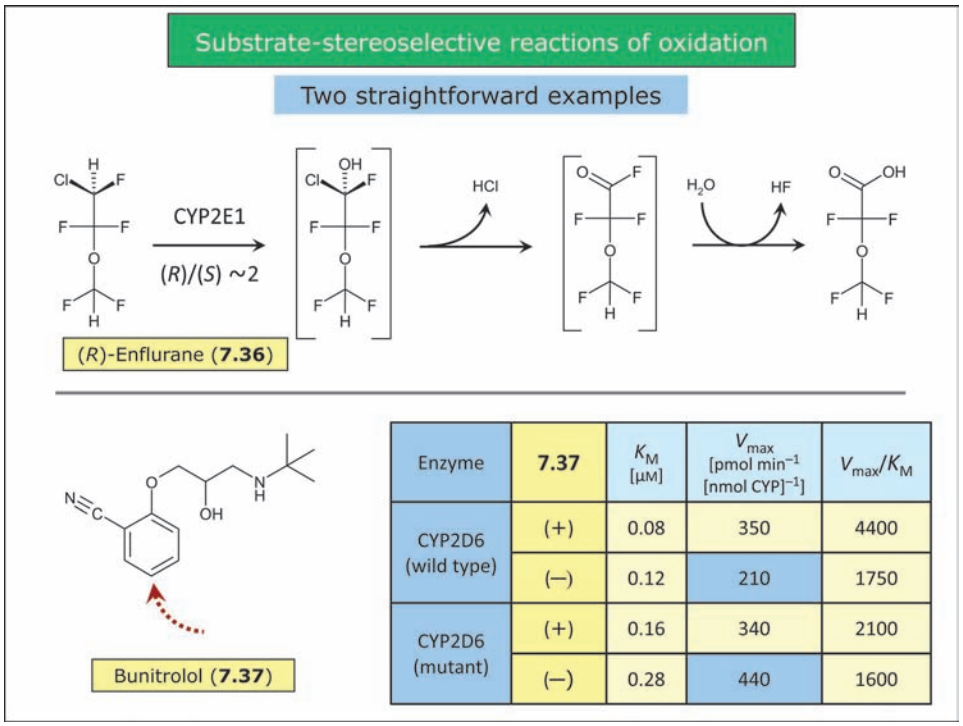
The *Figure* also shows a highly schematic representation of the published docking models [67]. Nonpolar residues are in blue, and polar ones in green. In both complexes, Gly142 and Gly143 form the oxyanion hole, while Ser211 and His468 are essential members of the catalytic triad [3][60]. A somewhat tighter binding of the nonpolar residues around the phenyl and piperidyl rings of the *levo*-form might explain in part its better affinity. Similarly, a somewhat tighter packing of the target ester group of the *levo*-form might explain its higher turnover number.



Substrate	Wild type			G60A Mutant			I106A/F132A/H257Y Mutant		
	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M
(S_P,S_C)-**7.33**	2.4	0.04	0.02	2.8	0.02	0.01	1.7	2.1	1.2
(R_P,R_C)-**7.33**	0.34	48	140	0.8	120	150	1.1	0.3	0.3
(S_P,R_C)-**7.33**	0.78	0.3	0.38	10	7.0	0.7	1.2	11	9
(R_P,S_C)-**7.33**	0.42	4.8	11	0.6	33	55	4.5	0.3	0.07

Fig. 7.15. This *Figure* deals not with a drug but with an *organophosphonate diester*, **7.33**, whose four stereoisomers were prepared as model compounds of *insecticides* and of the chiral warfare agents *sarin* (**7.34**) and *soman* (**7.35**) [70]. Such poisonous xenobiotics act (and kill) by the irreversible inhibition of acetylcholinesterase. Their detoxification is thus a topic of prime significance, both in the animal body [71][72] and in the environment where bacterial phosphoric triester hydrolases (EC 3.1.8) and particularly *phosphotriesterase* (EC 3.1.8.1) play a major role [73].

Here, bacterial phosphotriesterase (the *wild type*) was compared with *two mutants* selected, based on existing results and obtained by site-directed mutagenesis [70]. One mutant had residue 60 changed from glycine to alanine (Gly60Ala≡*G60A*), while the other carried three point mutations (Ile106Ala/Phe132Ala/His257Tyr≡*I106A/F132A/H257Y*). The model compound **7.33** shows close structural analogy with sarin (**7.34**) and soman (**7.35**), and has two stereogenic centers like the latter, the P-atom and a C-atom in the pinacolyl side chain. Globally, the results show that substrate enantioselectivity is due mainly to the catalytic step (*i.e.*, k_{cat}) rather than the binding step. The wild-type enzyme shows a strong preference for the (R_P,R_C)-stereoisomer due to a high turnover number. The second best substrate is its (R_P,S_C)-epimer, while the (S_P)-configured isomers are poor substrates. The same ranking was seen with the G60A mutant, with the interesting finding that the turnover number (k_{cat}) of the (R_P,S_C)-isomer was improved fivefold. In contrast, the *triple mutant behaved differently*, its best substrate being the (S_P,R_C)-stereoisomer, followed by the (S_P,S_C)-isomer.



Enzyme	7.37	K_M [μM]	V_{max} [pmol min^{-1} [nmol CYP]$^{-1}$]	V_{max}/K_M
CYP2D6 (wild type)	(+)	0.08	350	4400
	(−)	0.12	210	1750
CYP2D6 (mutant)	(+)	0.16	340	2100
	(−)	0.28	440	1600

Fig. 7.16. *Redox reactions* and particularly *oxidations* catalyzed by cytochromes P450 (CYPs) have been frequently investigated for their substrate stereoselectivity, as illustrated in this and the following *Figures*. To begin with medicinal compounds undergoing a single major metabolic reaction, the volatile anesthetic *enflurane* (**7.36**) is of interest given its CYP2E1-catalyzed route of *simultaneous pharmacological inactivation* and *toxification* through metabolic activation. Indeed, hydroxylation at C(2) results in the liberation of chloride and the formation of a highly reactive acyl fluoride. The latter is rapidly inactivated by H_2O to the corresponding carboxylic acid, a process during which inorganic fluoride is liberated, which has been implicated in *occasional nephrotoxicity*. Even more serious is the acylation of liver proteins by the acyl fluoride, producing antigens which may cause *hepatitis or even hepatic necrosis*. There was thus an incentive to investigate the substrate enantioselectivity of the reaction, but the approximately twofold slower oxidation of the (*S*)-enantiomer [74] appears too modest to support the safer use of this single enantiomer.

The *β*-blocking agent *bunitrolol* (**7.37**) tells another story. This compound undergoes hydroxylation in the *para*-position to phenol as its primary metabolic reaction. This reaction is catalyzed by CYP2D6, with the (+)-enantiomer being the better substrate, demonstrating a two- to threefold higher catalytic efficiency, as shown here [75]. A Val374Met mutant had twofold decreased *catalytic efficiency* toward the (+)-isomer, but there was no change toward the (−)-isomer. Furthermore, the *(+)/(−)-enantioselectivity of the reaction was reversed* when expressed in V_{max} values (1.7 for the wild-type enzyme, and 0.76 for the mutant). This again points to the determining role played by the structure of the catalytic site in influencing both binding and transition state.

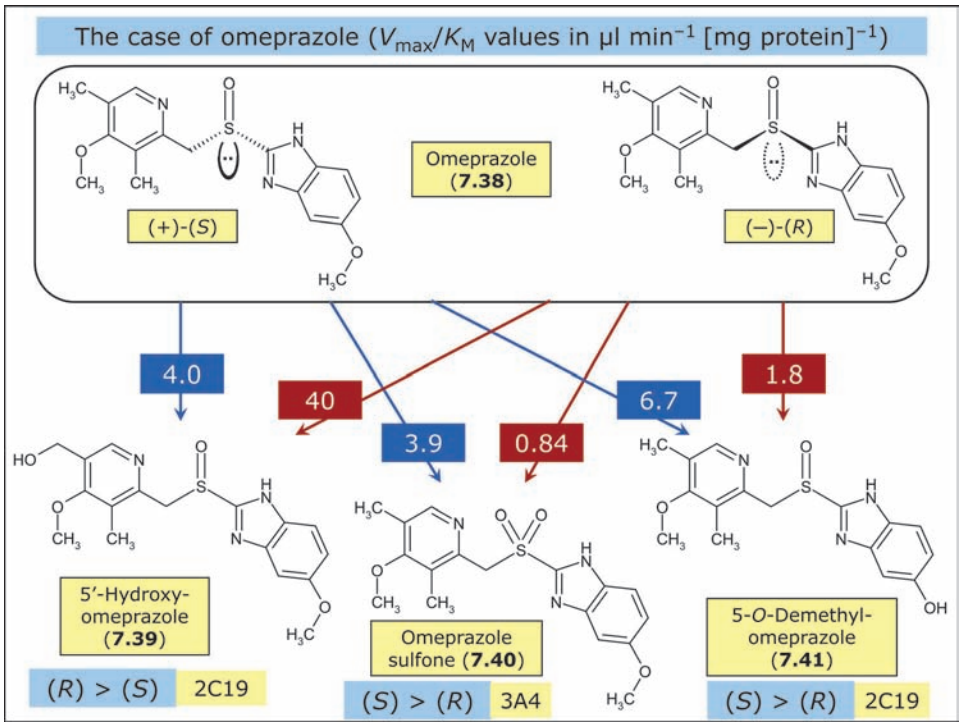


Fig. 7.17. The formation of *several metabolites from two stereoisomeric substrates* may result in a complex metabolic pattern, as illustrated in this and the next *Figure*. We begin with *omeprazole* (**7.38**), a proton pump inhibitor used to treat various acid-related gastrointestinal disorders [76]. This drug has additional relevance, since its chirality is due to an asymmetrically substituted sulfoxide group, with the (*S*)-enantiomer being a successful drug known as *esomeprazole*.

The three main metabolites of omeprazole are *5′-hydroxyomeprazole* (**7.39**), *omeprazole sulfone* (**7.40**), and 5-O-*demethylomeprazole* (**7.41**), all of which are formed by cytochrome P450 enzymes with marked selectivity. The number in the blue and red boxes are the V_{max}/K_M values measured in separate incubations of the enantiomers in human liver preparations. As shown, the formation of two metabolites, **7.40** and **7.41**, occurred with a marked preference for (*S*)-omeprazole, while the reverse enantioselectivity was observed in the formation of **7.39**. From an enzymatic viewpoint, incubations with nine expressed and major human CYP enzymes showed that *CYP2C19* was the main contributor to the formation of metabolites **7.39** and **7.41** from both (*R*)- and (*S*)-omeprazole, whereas *CYP3A4* was the main catalyst of (*R*)- and (*S*)-omeprazole sulfoxide oxygenation.

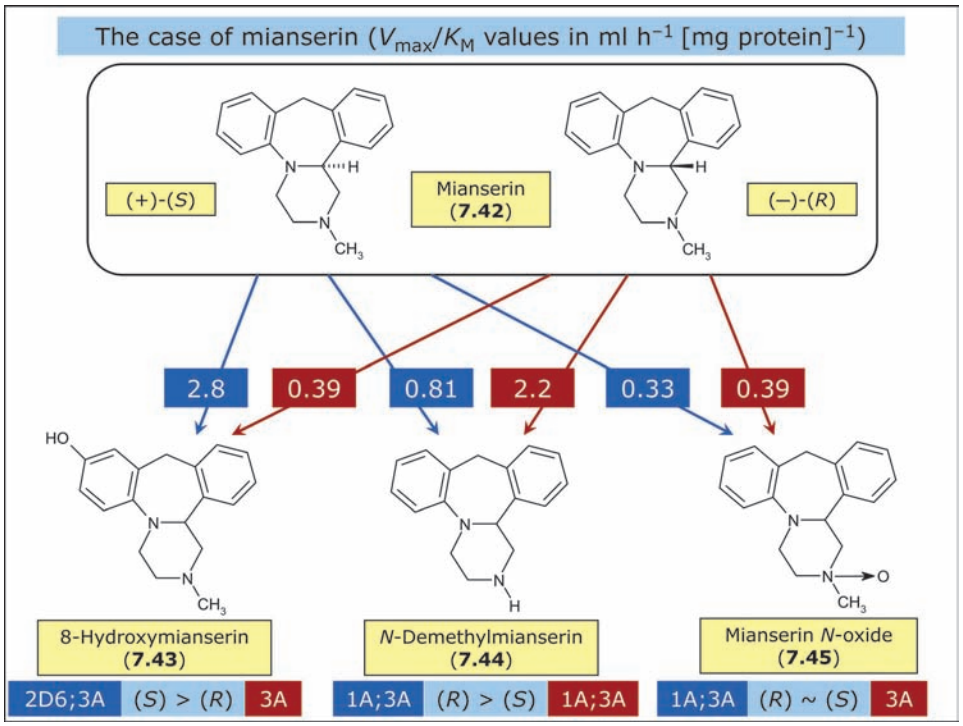


Fig. 7.18. The antidepressant drug *mianserin* (**7.42**) is used as the racemate, with the (*S*)-enantiomer contributing most of the *in vivo* activity, while the (*R*)-enantiomer was associated with higher cytotoxicity toward human leucocytes following *N*-demethylation. Here again, three major phase-I metabolites have been characterized, two involving *C*-oxidation and one *N*-oxygenation [77][78]. When the two enantiomers were incubated separately with human liver microsomes, the formation of *8-hydroxymianserin* (**7.43**) was selective for the (*S*) isomer, whereas the opposite was true for the formation of N-*demethylmianserin* (**7.44**). But one of the reactions lacked enantioselectivity, namely the formation of *mianserin* N-*oxide* (**7.45**) [77].

What also distinguishes this example from the previous one is the fact that for two of the reactions, different CYP enzymes were the major catalysts of (*R*)- and (*S*)-mianserin metabolism. Thus, CYP3A was a major contributor to all reactions, but it shared this role with CYP2D6 in the 8-hydroxylation of (*S*)-mianserin, and with CYP1A in the *N*-demethylation of both enantiomers and in the *N*-oxygenation of (*R*)-mianserin.

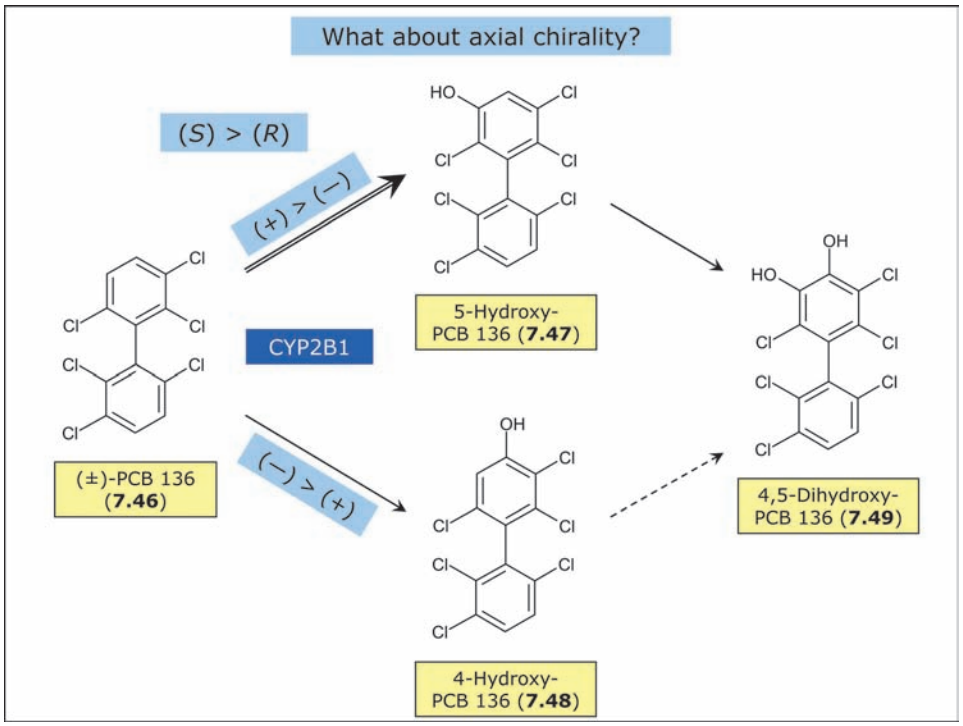


Fig. 7.19. All above examples are based on central chirality. However, axial chirality may also lead to enantioselectivity, as illustrated here with *2,2′,3,3′,6,6′-hexachloro-1,1′-biphenyl* (PCB 136; **7.46**). Axial chirality was discussed in *Part 3* where the high configurational stability of PCB 136 was illustrated, and in *Part 5* where its enantioselective toxic potential was mentioned.

PCB 136 and other halogenated biphenyls are substrates of cytochromes P450, in particular CYP2B1, resulting in the production of hydroxylated metabolites. Thus, PCB 136 incubated with induced rat liver microsomes was metabolized to *5-hydroxy-PCB 136* (**7.47**) as the major metabolite, *4-hydroxy-PCB 136* (**7.48**) as a minor one, and 4,5-dihydroxy-PCB 136 (**7.49**) as a very minor one [79]. The enantioselectivity in the generation of the two monohydroxylated metabolites was also determined, showing 5-hydroxy-PCB 136 (**7.47**) to be formed mainly from (+)-PCB 136 with a (+)/(−) ratio of *ca.* 2 : 1. In contrast, the formation of the minor metabolite 4-hydroxy-PCB 136 (**7.48**) was selective for (−)-PCB 136, with a (+)/(−) ratio of *ca.* 0.6 : 1. The absolute configuration of the enantiomers of PCB 136 is unknown at present, so that the enantioselectivity of their CYP-catalyzed oxidations cannot be interpreted in mechanistic terms. However, these metabolic results become significant in a toxicological perspective, as only the (−)-enantiomer of PCB 136 causes adverse neurodevelopmental effects through the sensitization of ryanodine receptors (see *Part 5*).

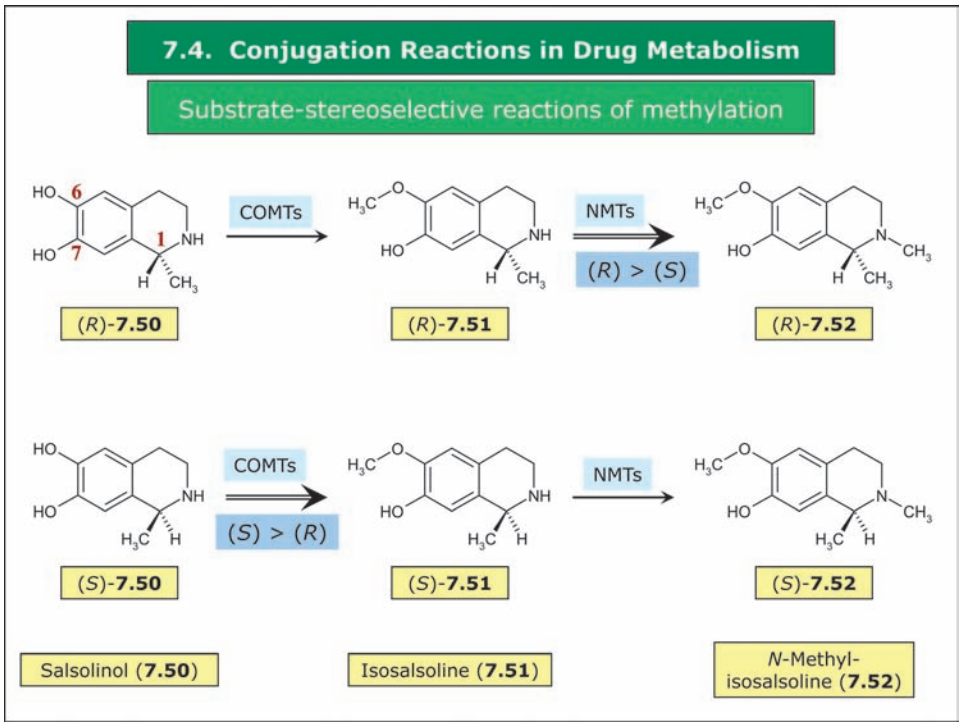
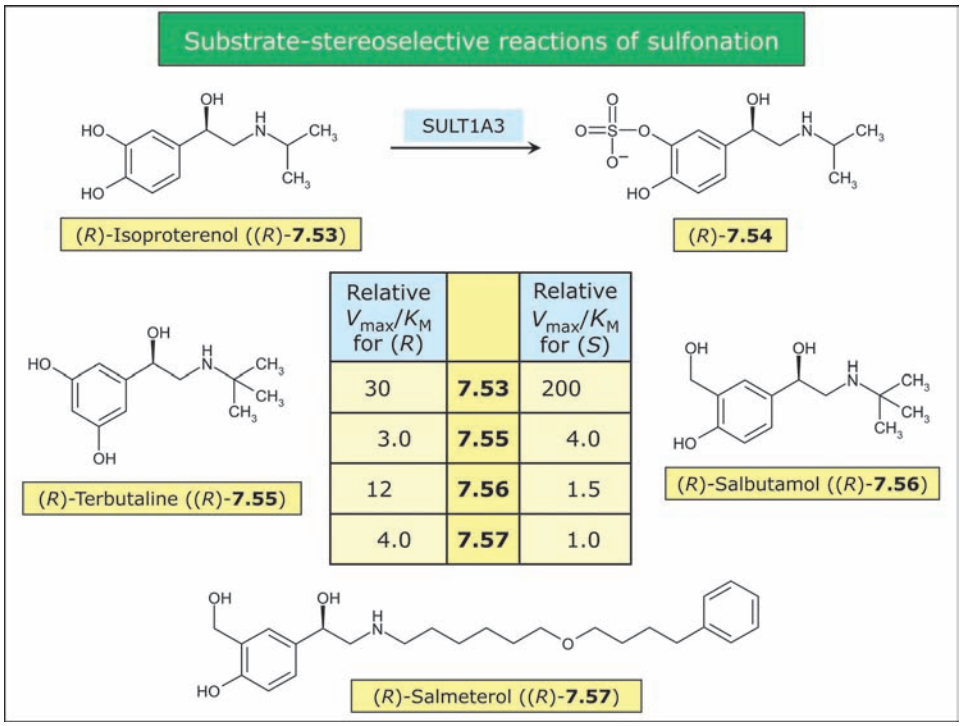


Fig. 7.20. Moving to *reactions of conjugation*, we should recall that these *a*) involve the coupling of a substrate molecule to an *endogenous molecule b*), which is *usually polar*, *c*) of 'medium' molecular weight (*ca. 100–300* Da), and *d*) linked to a *coenzyme*, while *e*) the reaction itself is catalyzed by a *transferase*. Criterion *a* is essential, whereas the others are not, and exceptions occur [4][80].

Reactions of methylation occur at catechol, amino, and thiol groups. The moiety transferred (a Me group) is obviously achiral, and the elements of chirality in substrates are left intact. *Salsolinol* (**7.50**), the substrate selected here, is both an endogenous compound found in mammals, where it is produced by the coupling of dopamine and acetaldehyde, and an environmental compound of plant origin which finds its way in foods and beverages. As a catechol, it is easily *O*-methylated by *catechol* O-*methyltransferase* (COMT; EC 2.1.1.6) to yield 6-*O*-methylsalsolinol (*isosalsoline*; **7.51**) and the 7-*O*-Me regioisomer not considered here. The formation of isosalsoline is *substrate-enantioselective* in that (*S*)-salsolinol is the preferred substrate of 6-*O*-methylation [81].

The significance of this example lies in the fact that the enantiomers of isosalsoline are themselves substrates of a methylation reaction, this time an N-*methylation* catalyzed by *amine* N-*methyltransferase* (NMT; 2.1.1.49). In this case, the preferred substrate in the formation of *N*-methylisosalsoline (**7.52**) is the (*R*)-enantiomer [82].



Relative V_{max}/K_M for (*R*)		Relative V_{max}/K_M for (*S*)
30	**7.53**	200
3.0	**7.55**	4.0
12	**7.56**	1.5
4.0	**7.57**	1.0

Fig. 7.21. Like reactions of methylation, conjugations with sulfate (*i.e.*, *sulfoconjugations*) involve the transfer of an achiral moiety and, seldom if ever, either create or erase a pre-existing element of chirality in the substrate. Sulfoconjugations are catalyzed by various *sulfotransferases* (SULTs) in sub-subclass EC 2.8.2. The cofactor involved is 3′-phosphoadenosine 5′-phosphosulfate (*PAPS*), target groups being phenols, alcohols, hydroxylamines, and certain amines [4][80]. Here, we look at four β_2-*receptor agonists* used in the treatment of asthma, namely *isoproterenol* (**7.53**; isoprenaline), *terbutaline* (**7.55**), *salbutamol* (**7.56**), and *salmeterol* (**7.57**). *In vitro* studies using human intestinal cytosol and recombinant human SULT1A3 [83] have confirmed their effective sulfonation (in the case of **7.53**, leading to sulfate **7.54**). While all the drugs examined had comparable V_{max} values, their affinities (K_M) showed marked structure and configuration dependence [84][85]. A more informative approach is thus to compare catalytic efficiencies (V_{max}/K_M), shown here more simply as relative catalytic efficiencies (rounded off values).

Isoproterenol (**7.53**) was clearly the best substrate, and its inactive (*S*)-enantiomer was sulfonated much more efficiently. The other three β_2-receptor agonists were comparatively poor substrates, with the active (*R*)-salmeterol and mainly (*R*)-salbutamol being the preferred enantiomers. Stated differently, substrate enantioselectivity was strongly influenced by molecular structure.

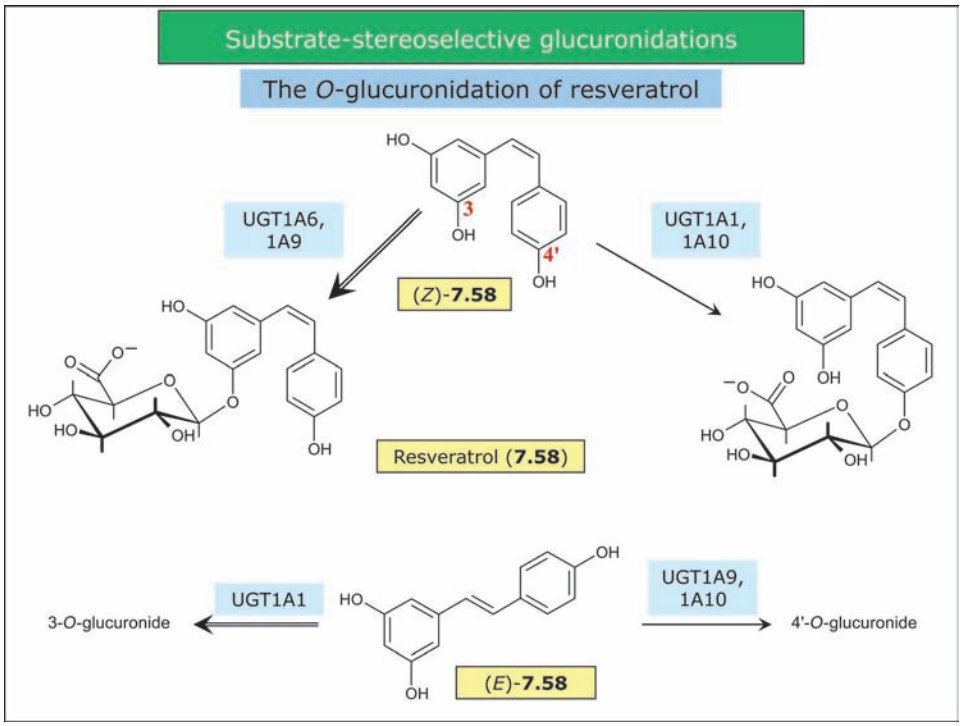


Fig. 7.22. Glucuronidations are catalyzed by UGT-glucuronosyltransferases (UGTs; EC 2.4.1.17), a superfamily of proteins of which more than 20 are active in humans [4][80]. These enzymes catalyze the transfer and binding of glucuronic acid to alcohols, phenols, carboxylic acids, hydroxylamines, amines, thiols, thioacids, and others. Glucuronic acid contains five stereogenic centers, meaning that the glucuronidation of a pair of enantiomers will yield a pair of corresponding epimeric conjugates, a bonus for bioanalysts.

The *O*-glucuronidation of *natural phenols* such as flavonoids has an evolutionary rationale [86]. The example selected here is that of *resveratrol* (**7.58**), a natural phenol found in a variety of plant sources, most notably grapes, and known for its antioxidant, lipid-lowering, cardioprotective, and chemopreventive activities. Resveratrol is an achiral molecule occurring as the (*E*)- and (*Z*)-diastereoisomers (also known as *trans*- and *cis*-resveratrol, resp.). Its *O*-glucuronidation occurs regioselectively in the 3- and 4′-position, and also shows a marked substrate diastereoselectivity depending on the enzymes and tissues involved [87–90]. The selectivities shown here ((*Z*)-3-*O* > (*E*)-3-*O* > (*Z*)-4′-*O* ~ (*E*)-4′-*O*) are those observed in incubations with human liver microsomes [87].

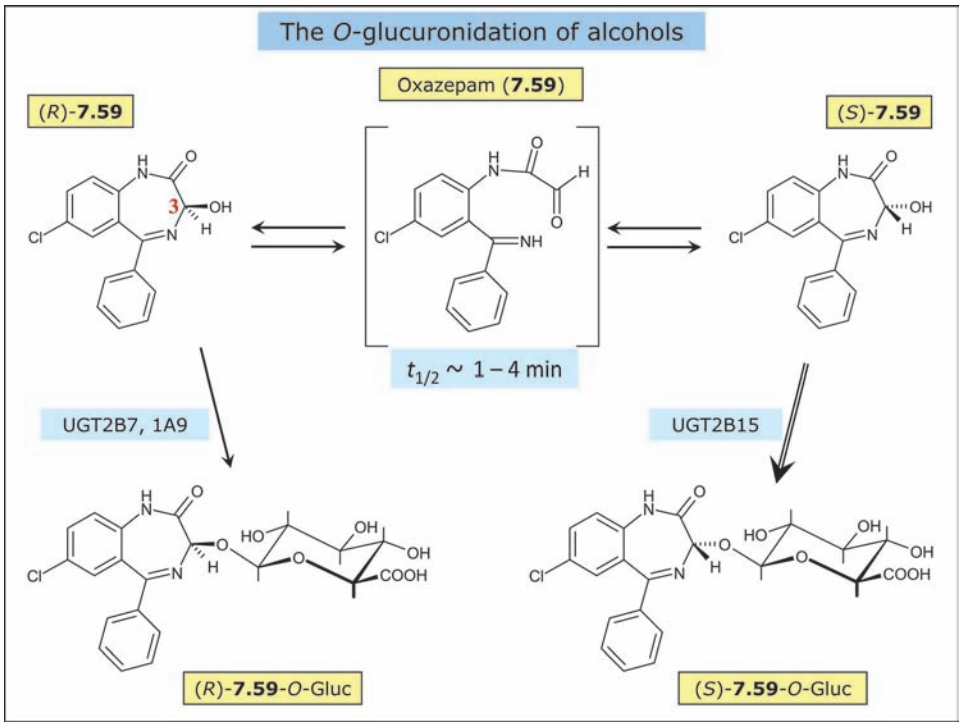


Fig. 7.23. The literature contains a large number of publications documenting the substrate-enantioselective O-*glucuronidation of alcohols*, as exemplified by a detailed study of the structure–metabolism relationships of enantiomeric bi- and tricyclic analogs of benzyl alcohol [91]. Here, we look at a rather unusual example, namely the sedative-hypnotic drug *oxazepam* (**7.59**). Its stereogenic center C(3) is highly unstable in protic environments, such that the enantiomers interconvert with an estimated *half-life of 1 – 4 min* under physiological conditions of pH and temperature [92][93]. The mechanism is pH-dependent and is assumed to involve ring⇌chain tautomerism featuring an achiral open form; deprotonation at C(3) to form an intermediate resonance-stabilized anion is another possibility.

Despite this fast nonenzymatic enantiomeric interconversion, incubations with human liver microsomes did show a clear *substrate enantioselectivity* for (*S*)-oxazepam. Furthermore, individual UGTs acting on this substrate were also enantioselective, since UGT2B15 was specific for (*S*)-oxazepam, whereas UGT1A9 and 2B7 glucuronidated (*R*)-oxazepam [94][95]. Glycosylation of the HO–C(3) group confers *configurational stability as glucuronides*, which are easily confirmed as *epimers*.

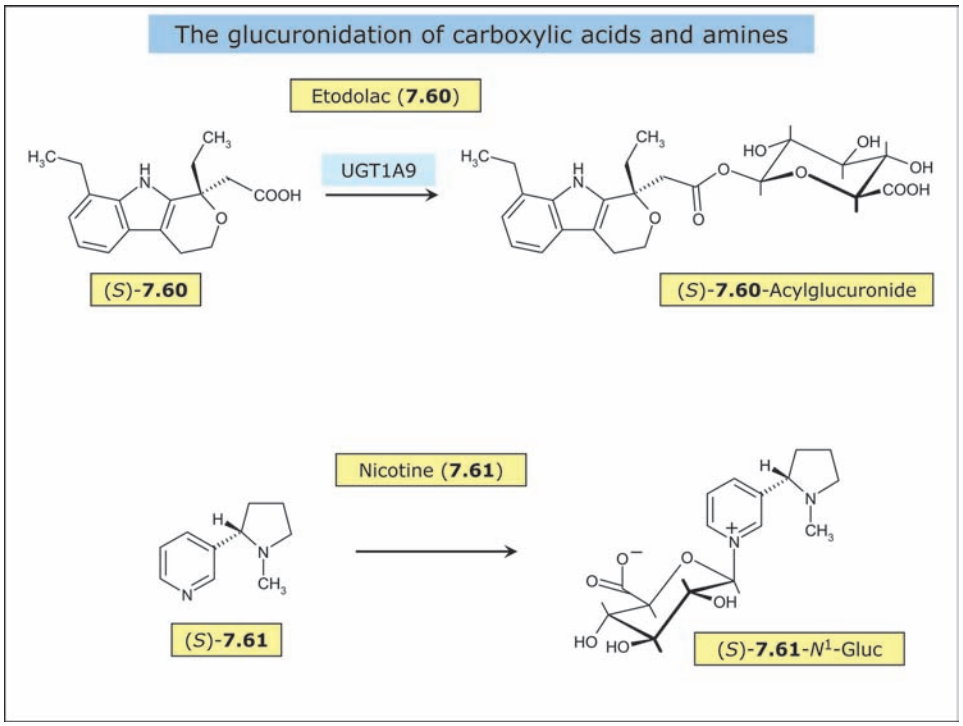


Fig. 7.24. We conclude this *Part* with two distinctive examples of glucuronides whose properties set them aside from the more common *O*-glucuronides of alcohols and phenols. Our first example is that of acyl glucuronides whose *significance* is now recognized [4][80][96]. These metabolites are quite reactive due to the combination of *an ester and an acetal function*, leading to potential immunogenicity and antigenicity following direct *transacylation of proteins*. A number of chiral nonsteroidal anti-inflammatory drugs (NSAIDs) readily form acyl glucuronides, adding a further dimension to their already complex metabolic behavior [97]. Thus, the NSAID *etodolac* (**7.60**) is marketed as the racemate, although the active form is the *(*S*)-enantiomer* shown in the *Figure*. This drug is extensively conjugated in humans to a rather reactive acyl glucuronide, the reaction in human liver microsomes being about fourfold faster for the active (*S*)-**7.60** than for its enantiomer [98]. Human UGT1A9 was the major enzyme involved in (*S*)-etodolac glucuronidation, with low contributions from 1A10 and 2B7. With the exception of 2B7, individual UGTs showed very low activity toward (*R*)-etodolac.

A second group of glucuronides are formed from various *tertiary amines* including aliphatic, alicyclic, or *pyridine-type* amines [99]. These *N*-glucuronides are special in the sense that, as quaternary amines, they contain a permanent positive charge in addition to the negative charge carried by the carboxylate, at least under physiological conditions; they are thus *zwitterions* [100]. A typical substrate is *nicotine* (**7.61**) which is N^+-glucuronidated by human UGT1A4 and UGT1A9. In human liver microsomes, a marked substrate enantioselectivity was seen such that the *natural (*S*)-enantiomer*

showed a fourfold higher catalytic efficiency than (*R*)-nicotine [101–103]. Nicotine N^1-glucuronide is found in the urine of smokers, with large quantitative differences depending on the relative activities of the many enzymes involved in the metabolism of nicotine.

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