CHAPTER 1

Biocatalysis and Green Chemistry

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1.1 INTRODUCTION TO SUSTAINABLE DEVELOPMENT AND GREEN CHEMISTRY

The publication in 1987 of the report *Our Common Future* by the World Commission on Environment and Development, otherwise known as the Brundtland Report [1], marked the advent of the concept of sustainable development. The report recognized the necessity for industrial and societal development to provide a growing global population with a satisfactory quality of life, but that such development must also be sustainable over time. Sustainable development was defined as *development that meets the needs of the present generation without compromising the needs of future generations to meet their own needs*. In order to be sustainable, it must fulfill two conditions: (i) natural resources should be used at rates that do not unacceptably deplete supplies over the long term, and (ii) residues should be generated at rates no higher than can be assimilated readily by the natural environment [2]. It is abundantly clear, for example, that a society based on nonrenewable fossil resources—oil, coal, and natural gas—is not sustainable in the long term. Sustainability consists of three components: societal, ecological, and economic, otherwise referred to as the three P's—people, planet, and profit.

At the same time, in the mid-1980s, there was a growing concern regarding the copious amounts of waste being generated by the chemical industry. Clearly, a paradigm shift was needed from traditional concepts of reaction efficiency and selectivity, which focus largely on chemical yield, to one that assigns value to maximization of raw materials utilization, elimination of waste, and avoiding the use of toxic and/or hazardous substances [3]. By the same token, there was a pressing need for alternative, cleaner chemistry in order to minimize these waste streams. It led to the emergence of the concepts of waste minimization, zero waste plants, and green chemistry [4]. The latter can be succinctly defined as [5]:

> Green chemistry efficiently utilizes (preferably renewable) raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products.

Originally it was referred to as "clean chemistry" [6]. The now widely accepted term "green chemistry" was introduced in the mid-1990s by Anastas and colleagues [7] of the US Environmental Protection Agency (EPA). The guiding principle is *benign by design* [8] as embodied in the 12 principles of green chemistry of Anastas and Warner:

Green Biocatalysis, First Edition. Edited by Ramesh N. Patel.

The 12 principles of green chemistry are as follows:

1. Waste prevention instead of remediation
2. Atom efficiency
3. Less hazardous materials
4. Safer products by design
5. Innocuous solvents and auxiliaries
6. Energy efficient by design
7. Preferably renewable raw materials
8. Shorter synthesis (avoid derivatization)
9. Catalytic rather than stoichiometric reagents
10. Design products for degradation
11. Analytical methodologies for pollution prevention
12. Inherently safer processes

Green chemistry eliminates waste at source; that is, it is primary pollution prevention rather than end-of-pipe waste remediation, as is inherent in the first principle of green chemistry: prevention is better than cure. Since the mid-1990s, the concept of green chemistry has been widely embraced in both industrial and academic circles [9]. One could say that sustainable development is our ultimate common goal and green chemistry is a means to achieving it.

1.2 GREEN CHEMISTRY METRICS

In order to know whether one process or product is greener than another one, we need meaningful metrics to measure greenness. The most widely accepted metrics of the environmental impact of chemical processes are, probably not coincidentally, the two most simple ones: the E factor [3–6, 10, 11], defined as the mass ratio of waste to desired product, and the atom economy (AE), defined as the molecular weight of the desired product divided by the sum of the molecular weights of all substances produced in the stoichiometric equation, expressed as a percentage [12, 13]. Knowledge of the stoichiometric equation enables one to predict, without performing any experiments, the theoretical amount of waste that will be formed. In Figure 1.1, for example, the AE of the classical chlorohydrin route to ethylene oxide is compared with that of catalytic oxidation with dioxygen. It is interesting to note that the former process produces, on a weight basis, more calcium chloride than ethylene oxide.

The AE is a theoretical number that is based on the assumption that a chemical yield of 100% of the theoretical yield is obtained and that reactants are used in exactly stoichiometric amounts. Furthermore, it disregards substances, such as solvents and acids or bases used in work-up, which do not appear in the stoichiometric equation. The E factor, in contrast, is the actual amount of waste produced in the process, defined as everything but the desired product. It takes the chemical yield into account and includes all reagents, solvent losses, all process aids, and, in principle, even the energy consumed.

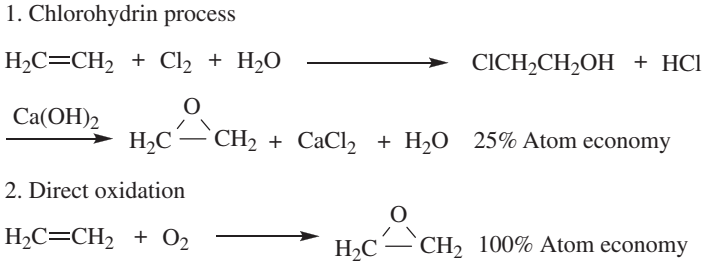

FIGURE 1.1
Atom efficiencies of two processes for ethylene oxide.

Originally [3] water was excluded from the calculation of the E factor as it was thought that its inclusion would lead to exceptionally high E factors in many cases and make meaningful comparisons of processes difficult. There is a definite trend, however, especially in the pharmaceutical industry, toward the inclusion of water in the E factor. The ideal E factor is zero, that is, zero waste. A higher E factor means more waste and, consequently, greater negative environmental impact. Alternatively, one can view the E factor as kilograms of raw materials minus kilograms of desired product, divided by kilograms of product out. It is easily calculated from knowledge of the number of tons of raw materials purchased and the number of tons of product sold. This method of calculation automatically excludes water used in the process, but not the water formed. Typical E factors for various segments of the chemical and allied industries, which we originally estimated in 1992, indicated that the fine chemical and pharmaceutical industries created a substantial waste burden [3]. A modified version of the original presentation, in which the oil-refining segment has been replaced by basic petrochemical hydrocarbon feedstocks, is shown in Table 1.1. This table also contains ranges of (average) annual product tonnages, which allow the annual tonnages of waste to be estimated. Such calculations could have been done in the original presentation, but we refrained from doing so because the relatively low figures for the annual waste tonnages for the pharmaceutical industry could be construed as a reason for inaction on the part of this industry segment, whereas E factors clearly show the need for action.

The substantial increase in E factors on moving downstream from bulk chemicals to fine chemicals and, particularly, pharmaceuticals is a reflection of the increasing molecular complexity of the products and associated multistep syntheses, which can be expected to generate more waste. Consequently, waste generation can be reduced by developing processes that are more step economic as advocated by Wender [14]. In bulk chemicals and basic hydrocarbon feedstock manufacture, in contrast, target molecules are simpler and require a smaller number of steps for their synthesis. This is not the whole story, however. The high E factors in pharma and fine chemicals are also a direct consequence of the widespread use of stoichiometric inorganic and organic reagents in these industry segments. In bulk chemicals manufacture, in contrast, because of the enormous production volumes, the use of stoichiometric reagents is economically prohibitive. We also note that E factors for the production of therapeutic proteins (biopharmaceuticals) on a commercial scale are even higher [15]. The E factor has been widely adopted by the chemical industry—in particular by the pharmaceutical industry [16], as a useful metric for assessing the environmental impact of manufacturing processes [17, 18] and has been shown to be predictive of reductions in manufacturing costs [19].

The number of green metrics subsequently proliferated [20–23]. They can be divided into two types: (i) metrics that are a refinement of the AE concept and (ii) metrics that are variations of the E factor (see Figure 1.2). Examples of the former are reaction mass efficiency (RME) and carbon efficiency (CE) introduced by Constable and coworkers [24] at GlaxoSmithKline (GSK). The RME is defined as the mass of

TABLE 1.1 E Factors in the Chemical and Allied Industries

Industry Segment	Annual Product Tonnage	E Factor (kg waste/ kg product)	Total Annual Waste Tonnage
Basic petrochemicals (ethylene, propylene, butadiene, ethylbenzene)	10 000 000–100 000 000	~0.1	10 000 000
Bulk chemicals (propylene oxide, caprolactam)	10 000–1 000 000	<1 to 5	5 000 000
Fine chemicals (flavors and fragrances, cosmetic ingredients)	100–10 000	5 to >50	500 000
Pharmaceuticals	10–1 000	25 to >100	100 000

E factor

$$E = \frac{\text{Total mass of waste}}{\text{Mass of final product}}$$

Atom efficiency (AE)

$$AE\ (\%) = \frac{\text{m.w of product} \times 100}{\Sigma\ \text{m.w. of reactants}}$$

Mass intensity (MI)

$$MI = \frac{\text{Total mass used in a process}}{\text{Mass of product}}$$

Reaction mass efficiency (RME)

$$RME\ (\%) = \frac{\text{Mass of product C} \times 100}{\text{Mass of A} + \text{Mass of B}}$$

FIGURE 1.2
Green chemistry metrics.

product obtained divided by the total mass of reactants in the stoichiometric equation, expressed as a percentage. It is a refinement of the AE that takes the chemical yield of the product and the actual quantities of reactants used into account. A disadvantage compared to AE is the requirement for experimental data to calculate the RME, which, therefore, cannot be used for rapid analysis of different processes prior to experimental work being performed. The CE is similar to RME but takes only carbon into account, that is, it is the mass of carbon in the product obtained divided by the total mass of carbon present in the reactants.

An example of the second type is mass intensity (MI) [25], defined as the total mass of materials used in a process divided by the mass of product obtained, that is, MI = E factor + 1. An analogous metric, the effective mass yield (EMY), is defined as the mass of the desired product divided by the total mass of nonbenign reactants used in its preparation [26]. The EMY does not include so-called environmentally benign compounds, such as NaCl and acetic acid, but defining nonbenign is difficult and arbitrary.

The AE and E factor are complementary: the former can be used for a quick assessment, before conducting any experiments, while the latter is a measure of the total waste that is actually formed in practice. None of the alternative metrics offer any particular advantage over the AE and E factors for assessing how wasteful a process is. The ideal E factor is zero, which is a better reflection of the ultimate goal of zero waste manufacturing plants than the ideal MI of 1. Moreover, the E factor concept is mathematically simpler since *step E factor contributions are additive while step PMI contributions are not*, because the PMI does not discount the step product from the step mass balance [27].

1.3 ENVIRONMENTAL IMPACT AND SUSTAINABILITY METRICS

Sustainability encompasses the conservation of the Earth's natural resources and minimization of the effect of industrial activities on the health of its inhabitants and the natural environment in addition to economic viability. Green chemistry embodies essentially the same two elements: (i) efficient utilization of raw materials and elimination of waste and (ii) health, safety, and environmental aspects of chemicals and their manufacturing processes but without the economic component. However, the metrics discussed in the preceding section take only the mass of waste generated into account, whereas the environmental impact of this waste is also determined by its nature. Hence, we introduced [6] the term "environmental quotient" (EQ) to take the nature of the waste into account. EQ is the product of the E factor and an unfriendliness multiplier, Q. The latter is dependent on various factors such as toxicity, ease of recycling, etc., and can also be influenced by both the production volume and the location of the facilities. For example, the generation of 100–1000 tons per annum of sodium chloride is unlikely to present a waste problem, but 10 000 tons per annum, in contrast, may already present a disposal problem, thus warranting an increase in *Q*. Ironically, when very large quantities of sodium chloride are generated, the *Q* value could decrease again as recycling by electrolysis becomes viable, for example, in propylene oxide manufacture via the chlorohydrin route (see earlier). Hence, the *Q* value

of a particular waste will be determined by, *inter alia*, its ease of disposal or recycling. Generally speaking, organic waste is more easily remediated than inorganic waste, which can be important when considering the green metrics of biocatalytic processes.

Since the mid-2000's, several groups have addressed the problem of quantifying *Q*. For example, Eissen and Metzger [28] developed the Environmental Assessment Tool for Organic Syntheses (EATOS) software in which metrics related to health hazards and persistence and bioaccumulation and ecotoxicity were used to determine the environmental index of the input (substrates, solvents, etc.) and the output (product and waste). Similarly, Saling and coworkers at BASF [29–31] introduced eco-efficiency analyses, which took both economic and environmental aspects into account, including energy, raw materials, emissions, toxicity, hazards, and land use.

The basis for such an analysis is life-cycle assessment (LCA) [32, 33], which is used to assess the environmental impact and sustainability of products and processes within defined domains, for example, cradle to gate, cradle to grave, and gate to gate, on the basis of quantifiable environmental impact indicators, such as energy usage, global warming, ozone depletion, acidification, eutrophication, smog formation, and ecotoxicity, in addition to waste generated. Jessop and coworkers [34], for example, used a combination of eight LCA environmental impact indicators—acidification, ozone depletion, smog formation, global warming, human toxicity by ingestion and inhalation, persistence, bioaccumulation, and abiotic resource depletion—in a gate-to-gate assessment of the greenness of alternative routes to a particular product. The outcome of an LCA resembles an EQ in that it constitutes an integration of the amount of waste with quantifiable environmental indicators based on the nature of the waste.

1.4 SOLVENTS

Typically, solvents constitute more than half of the materials used in a chemical process to produce a drug substance [35]. Consequently, a major source of waste in chemicals manufacture, and an important contributor to high E factors in pharma, is solvent losses, which generally end up in the atmosphere or in groundwater. Moreover, there are health and/or safety issues associated with many traditional organic solvents, such as chlorinated hydrocarbons.

The FDA has issued guidelines [36] for solvent use in the pharmaceutical industry and divided them into four classes:

- Class 1 solvents should be avoided in the manufacture of drug substances because of their unacceptable toxicity or deleterious environmental effects. They include benzene and various chlorinated hydrocarbons.
- Class 2 solvents should be used only sparingly in pharmaceutical processes because of inherent toxicity. They include acetonitrile, dimethyl formamide, methanol, and dichloromethane.
- Class 3 solvents are regarded as less toxic and of lower risk to human health and are, hence, preferred. They include many lower alcohols, esters, ethers, and ketones.
- Class 4 solvents, for which no adequate data are available, include diisopropyl ether, methyl tetrahydrofuran, and isooctane.

Consequently, industrial attention is focused both on minimizing overall solvent use and in replacing many traditional organic solvents, such as chlorinated and aromatic hydrocarbons, by more environmental-friendly alternatives such as lower alcohols, esters, and some ethers such as methyl tert-butyl ether (MTBE). Several pharmaceutical companies have produced solvent selection guides to help their chemists in selecting greener, more sustainable solvents [37]. Pfizer, for example,

classified solvents into three categories: preferred, usable, and undesirable with an advice regarding substitution of undesirable solvents [16, 38]. Sanofi scientists [39] divided solvents into four categories based on safety, health, and environmental hazards and other industrial issues: (i) recommended, (ii) substitution advisable, (iii) substitution requested, and (iv) banned. Similarly, GSK has a similar guide, with two safety criteria, one health criterion, three environmental criteria including life cycle scoring, and additional red flags, for example, for solvents governed by regulations [40, 41]. Solvents derived from renewable feedstocks, such as ethanol, ethyl lactate, and methyl tetrahydrofuran [42], are becoming popular reaction media as they are seen as "natural" and sustainable.

In the original inventory of E factors of various processes, we assumed [3], if data were not available, that solvents would be recycled by distillation and that this would involve a 10% loss. However, this was probably overoptimistic, certainly for the pharma industry where the widespread use of different solvents for the various steps in multistep syntheses makes recycling difficult owing to cross contamination. The best solvent is no solvent, but if a solvent is needed, it should be safe to use and there should be provisions for its efficient removal from the product and reuse.

1.5 THE ROLE OF CATALYSIS

The waste generated in the manufacture of fine chemicals and pharmaceuticals is largely due to the use of stoichiometric inorganic and organic reagents that are partially incorporated or not incorporated into the product. Typical examples include oxidations with inorganic oxidants such as chromium (VI) salts, permanganates, manganese dioxide, and stoichiometric reductions with metals (Na, Mg, Zn, Fe) and metal hydrides ($LiAlH_4$, $NaBH_4$). Similarly, stoichiometric amounts of mineral acids (H_2SO_4, HF, and H_3PO_4) and Lewis acids ($AlCl_3$, $ZnCl_2$, BF_3) are major sources of waste. The solution is evident: the substitution of antiquated stoichiometric methodologies with cleaner catalytic alternatives [43–45]. This is true elegance and efficiency in organic synthesis [46]. For example, catalytic hydrogenation, oxidation, and carbonylation are highly atom-efficient processes. Similarly, the use of recyclable solid (heterogeneous) acids and bases as catalysts results in substantial reductions in waste in industrial organic synthesis [47, 48]. Indeed, several pharma companies have developed reagent guides for particular reaction types with the aim of improving the greenness and sustainability of their processes [41].

The ultimate in step and AE is the development of catalytic cascade processes whereby several catalytic steps are integrated in one-pot procedures without the need for isolation of intermediates [49]. Such "telescoping" of multistep syntheses into catalytic cascades has several advantages—fewer unit operations, less solvent and reactor volume, shorter cycle times, higher volumetric and space-time yields, and less waste (lower E factor)—that afford substantial economic and environmental benefits. Furthermore, coupling of reactions can be used to drive equilibria toward product, thus avoiding the need for excess reagents.

1.6 BIOCATALYSIS AND GREEN CHEMISTRY

Biocatalysis has many attractive features in the context of green chemistry and sustainable development:

1. The catalyst (an enzyme) is derived from renewable resources and is biocompatible (sometimes even edible), biodegradable, and essentially nonhazardous, that is, it fulfills the criteria of sustainability remarkably well.

2. Biocatalysis avoids the use of, and contamination of products by, scarce precious metals such as palladium, platinum, and rhodium. The long-term commercial viability of many "endangered" elements, such as various noble metals, is questionable. Moreover, the costs of removing traces of noble metals, to an acceptable level, from end products can be substantial.
3. Reactions are performed in an environmentally compatible solvent (water) under mild conditions (physiological pH and ambient temperature and pressure).
4. Reactions of multifunctional molecules proceed with high activities and chemo-, regio-, and stereoselectivities and generally without the need for functional group activation, protection, and deprotection steps required in traditional organic syntheses. This affords processes that are more step economic and more efficient in energy and raw material consumption, generate less waste, and are, therefore, both environmentally and economically more attractive than conventional routes.
5. As a direct result of the higher selectivities and milder reaction conditions, biocatalytic processes often afford products in higher purity than traditional chemical or chemo-catalytic processes.
6. Enzymatic processes (but not fermentations) can be conducted in standard multipurpose batch reactors and, hence, do not require any extra investment, for example, for high-pressure equipment.
7. Biocatalytic reactions are conducted under roughly the same conditions of temperature and pressure, and, hence, it is relatively easy to integrate multiple reactions into eco-efficient catalytic cascade processes [50].

In short, biocatalysis fits very well with the principles of green chemistry and sustainability. As Barry Commoner, the doyen of industrial ecology, observed [51]: "in nature there is no such thing as waste, everything is recycled." As shown in Table 1.2, biocatalysis conforms with 10 of the 12 principles of green chemistry and is not really relevant for the other two (principles 4 and 10), which are concerned with the design of safer, biodegradable products. Consequently, since the mid-1990's, biocatalysis has emerged as an important technology for meeting the growing demand for green and sustainable chemical manufacture [52, 53], particularly in the pharmaceutical industry [54, 55].

Thanks to advances in biotechnology and protein engineering techniques such as *in vitro* evolution [56], it is now possible to produce most enzymes for commercially

TABLE 1.2 Biocatalysis and the Principles of Green Chemistry

Green Chemistry Principles	Biocatalysis
1. Waste prevention	Enables more sustainable routes with significantly reduced waste
2. Atom economy	Enables more atom and step economic routes
3. Less hazardous syntheses	Generally low toxicity
4. Design for safer products	Not relevant
5. Safer solvents and auxiliaries	Usually performed in water or Class 3 solvents
6. Energy efficient	Mild conditions are conducive with energy efficiency
7. Renewable feedstocks	Enzymes are renewable
8. Reduce derivatization	Biocatalysis obviates the need for protection/deprotection
9. Catalysis	Enzymes are catalysts
10. Design for degradation	Not really relevant but enzymes themselves are biodegradable
11. Real-time analysis for pollution prevention	Can be applicable in biocatalytic processes
12. Inherently safer processes	Performed under mild and safe conditions

acceptable prices and to manipulate them such that they exhibit the desired properties with regard to, *inter alia*, substrate specificity, activity, selectivity, stability, and pH optimum [57, 58]. This has made it eminently feasible to optimize the enzyme to fit a predefined optimum process that is genuinely benign by design. Furthermore, the development of effective immobilization techniques has paved the way for optimizing the storage and operational stability and the recovery and recycling of enzymes [59]. In addition, the coimmobilization of two or more enzymes can afford multifunctional solid biocatalysts capable of catalyzing biocatalytic cascade processes [60].

Biocatalytic processes are performed with isolated enzymes or as whole-cell biotransformations. Isolated enzymes have the advantage of not being contaminated with other enzymes present in the cell. The use of whole cells, on the other hand, is less expensive as it avoids the separation and purification of the enzyme. In the case of dead cells, E factors of the two methods are essentially the same: the waste cell debris is separated before or after the biotransformation, respectively. In contrast, substantial amounts of waste biomass can be generated when using growing microbial cells in the fermentation processes. We note, however, that this waste is generally easy to dispose of, for example, as animal feed or can, in principle, be used as a source of energy for the process. Many fermentation processes also involve the formation of copious amounts of inorganic salts that may even be the major contributor to waste. E factors have generally not been calculated for fermentations, but published data [61] regarding mass balances can be used to calculate E factors. The E factor for the bulk fermentation product—citric acid, for example—is 1.4, which compares well with the E factor range of <1–5 typical of bulk petrochemicals. Interestingly, ca. 75% of the waste is accounted for by an inorganic salt, calcium sulfate. If water is included in the calculation, the E factor becomes 17. In contrast, small-volume fermentation processes for low-volume, high-added-value biopharmaceuticals can have extremely high E factors, even when compared with those observed in the production of small-molecule drugs. The fermentative production of recombinant human insulin [15], for example, involves an E factor of ca. 6600 and inclusion of water affords an astronomical E factor of 50 000! In contrast, biocatalysis with isolated enzymes tends to involve significantly higher substrate concentrations and combines a higher productivity with a lower water usage compared to fermentations.

1.7 EXAMPLES OF GREEN BIOCATALYTIC PROCESSES

1.7.1 A Chemoenzymatic Process for Pregabalin

Pfizer scientists have described [62] a second-generation chemoenzymatic process (Figure 1.3) for the manufacture of pregabalin, the active ingredient of the CNS drug Lyrica. It represented a dramatic improvement in process efficiency compared to earlier routes. The stereocenter was set early in the synthesis in accordance with the golden rule of chirotechnology [63], and the wrong enantiomer could be easily racemized and reused. The key enzymatic step was conducted with an inexpensive, readily available laundry detergent lipase at a staggering substrate concentration of 765 g/l. Organic solvent usage was dramatically reduced in a largely aqueous process. Compared to the first-generation manufacturing process, the new process afforded a higher yield and a fivefold reduction in the E factor from 86 to 17.

1.7.2 A Three-Enzyme Process for Atorvastatin Intermediate

Codexis scientists developed and commercialized a green-by-design, three-enzyme process for the synthesis of a key intermediate (Figure 1.4) in the manufacture of atorvastatin, the active ingredient of the cholesterol-lowering drug Lipitor [64, 65]. In the first step, ethyl-4-chloroacetoacetate undergoes highly enantioselective reduction

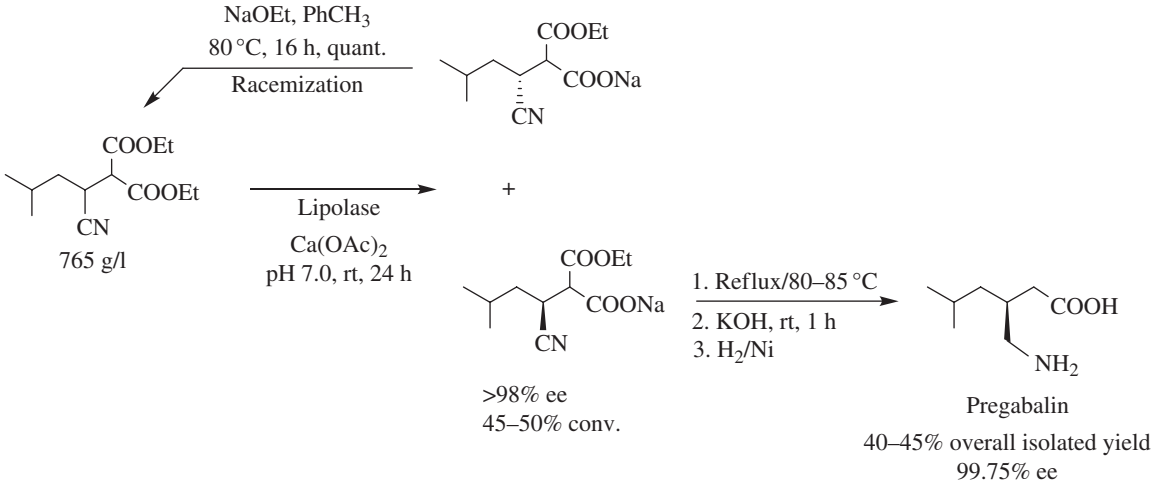

FIGURE 1.3
Chemoenzymatic process for pregabalin.

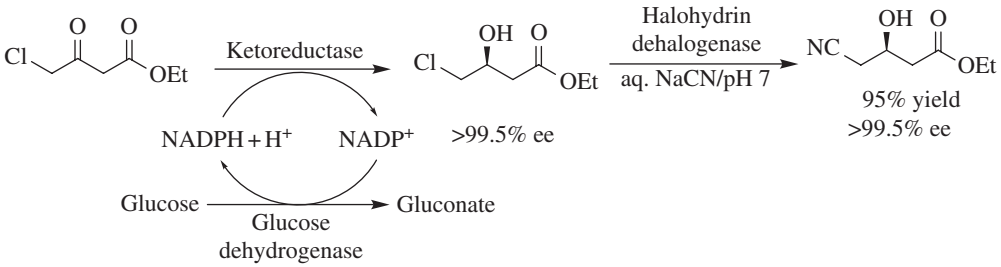

FIGURE 1.4
A two-step three-enzyme process for atorvastatin intermediate.

catalyzed by a ketoreductase (KRED). Cofactor regeneration was achieved with glucose as the hydrogen donor and an NADP-dependent glucose dehydrogenase (GDH) as the catalyst. The (*S*) ethyl-4-chloro-3-hydroxybutyrate product was obtained in 96% isolated yield and >99.5% ee. In the second step, a halohydrin dehalogenase (HHDH) was employed to catalyze a nucleophilic substitution of chloride by cyanide using HCN at neutral pH and ambient temperature.

All previous manufacturing routes to the hydroxynitrile product employed, as the final step, a standard S_N2 substitution of halide with cyanide ion in alkaline solution at elevated temperatures. This resulted in extensive by product formation owing to the base sensitivity of both substrate and product. Since the product is high-boiling oil, troublesome and expensive high-vacuum fractional distillation is required to recover product of acceptable quality, resulting in further yield losses and more waste. Hence, the key to designing an economically and environmentally attractive process was to conduct the cyanation reaction at ambient temperature and neutral pH using the enzyme, HHDH as the catalyst. Overall this afforded an elegant two-step, three-enzyme process for the hydroxynitrile product.

Unfortunately, the wild-type KRED and GDH exhibited prohibitively low activities, and large enzyme loadings were required to obtain an economically viable reaction rate. This resulted in troublesome emulsion formation and associated yield losses in downstream processing. Fortunately, the enzyme loadings could be drastically reduced by employing *in vitro* evolution via DNA shuffling [66] to improve the activity and stability of KRED and GDH. The GDH activity was improved by a factor of 13 and the KRED activity by a factor of 7 while maintaining the nearly perfect enantioselectivity (>99.5%) of the wild-type KRED. With the improved enzymes, the reaction was complete in 8 h with a substrate loading of 160 g/l and phase separation required <1 min, providing the chlorohydrin in >95% isolated yield and >99.9% ee.

Similarly, the activity of the wild-type HHDH in the nonnatural cyanation reaction was extremely low, and the enzyme exhibited severe product inhibition and poor stability under operating conditions. However, after many iterative rounds of DNA shuffling, the inhibition was largely overcome and the HHDH activity was increased more than 2500-fold compared to the wild-type enzyme.

The greenness of process was assessed according to the 12 principles of green chemistry:

Principle 1—waste prevention: The highly selective biocatalytic reactions afforded a substantial reduction in waste, and by avoiding by product formation, the need for yield-sacrificing fractional distillation was circumvented. The butyl acetate and ethyl acetate solvents, used in the extraction of the product from the aqueous layer in the first and second steps, respectively, were recycled with an efficiency of 85%. The E factor for the overall process is 5.8 if process water is excluded (2.3 for the reduction and 3.5 for the cyanation). If process water is included, the E factor for the whole process is 18 (6.6 for the reduction and 11.4 for the cyanation). The main contributors to the E factor (Table 1.3) are solvent losses (51%), sodium gluconate (25%), and the innocuous inorganic salts, NaCl and Na_2SO_4 (combined ca. 22%). The three enzymes and the NADP cofactor account for <1% of the waste. Furthermore, the main waste streams are aqueous and directly biodegradable.

Principle 2—AE: The use of glucose as the reductant for cofactor regeneration is cost-effective, but the AE is poor (45%). However, glucose is an inexpensive renewable raw material and the gluconate coproduct is fully biodegradable.

Principle 3—less hazardous chemical syntheses: The reduction reaction uses essentially nontoxic starting materials and avoids the use of potentially hazardous hydrogen and heavy metal catalysts obviating concern for their removal from waste streams and/or contamination of the product. While cyanide must be used in the second step, as in all practical routes to the product, it is used more efficiently (higher yield) and under less harsh conditions compared to previous processes.

Principle 4—design safer chemicals: This is not applicable as the hydroxynitrile product is the target molecule.

Principle 5—safer solvents and auxiliaries: Safe and environmentally acceptable ethyl acetate and butyl acetate are used, together with water, as cosolvent in the biocatalytic reduction reaction and extraction of the hydroxynitrile product. No auxiliaries are needed.

Principles 6 and 9—design for energy efficiency and catalysis: The process constitutes very efficient biocatalysis with turnover numbers of $>10^5$ for KRED and GDH and $>5\times10^4$ for HHDH. In contrast with previous processes, which employ elevated temperatures for the cyanation step and high-pressure hydrogenation

TABLE 1.3 E Factor of the Process for Atorvastatin Intermediate

Waste	Quantity (kg/kg product)	% of E (Excl. Water)	% of E (Incl. Water)
Substrate losses (8%)	0.09	<2	<1
Triethanolamine	0.04	<1	<1
NaCl and Na_2SO_4	1.29	22	ca. 7
Na-gluconate	1.43	ca. 25	ca. 9
BuOAc (85% recycle)	0.46	ca. 8	ca. 3
EtOAc (85% recycle)	2.50	ca. 43	ca. 14
Enzymes	0.023	<1	<1
NADP	0.005	0.1	<0.1
Water	12.250	—	67
Total waste (E factor)	5.8 kg (18 with H_2O)		

for the reduction step, both steps in the biocatalytic process are run at or close to ambient temperature and pressure and pH7, and the very high energy demands of high-vacuum distillation are dispensed with altogether, resulting in substantial energy savings.

Principles 7 and 10—the use of renewable feedstocks and design for degradation: The enzyme catalysts and the glucose cosubstrate are derived from renewable raw materials and are completely biodegradable. The by-products of the reaction are gluconate, NADP (the cofactor), residual glucose, enzyme, and minerals, and the waste water is directly suitable for biotreatment.

Principle 8—reduce derivatization: The process avoids derivatization steps, that is, it is step economic and involves fewer unit operations than earlier processes, most notably by obviating the troublesome product distillation or bisulfite-mediated separation of dehydrated byproducts.

Principles 11 and 12—real-time analysis for pollution prevention and inherently safer chemistry: The reactions are run in pH-stat mode at neutral pH by computer-controlled addition of base. Gluconic acid generated in the first reaction is neutralized with an aq. NaOH, and HCl generated in the second step is neutralized with feed-on-demand aq. NaCN, regenerating HCN ($pK_a \sim 9$) *in situ*. This minimizes the overall concentration of HCN, affording an inherently safer process. The pH and the cumulative volume of added base are recorded in real time.

In short, the Codexis process is an excellent example of a *benign by design* biocatalytic process for the synthesis of an important pharmaceutical intermediate whereby successful commercialization is enabled by employing modern protein engineering to optimize enzyme performance.

1.7.3 Enzymatic Synthesis of Sitagliptin

Another relevant example is provided by the enzymatic synthesis of the antidiabetic, sitagliptin, which was codeveloped by Merck and Codexis workers [67] to replace a rhodium-catalyzed, high-pressure, asymmetric hydrogenation of an enamine. It involves an overall enantioselective reductive amination of a ketone using an (*R*)-transaminase-catalyzed reaction with isopropylamine (Figure 1.5). The starting point was an (*R*)-selective transaminase, which showed no activity with the ketone substrate. *In silico* studies were employed to identify what was needed to be able to fit the

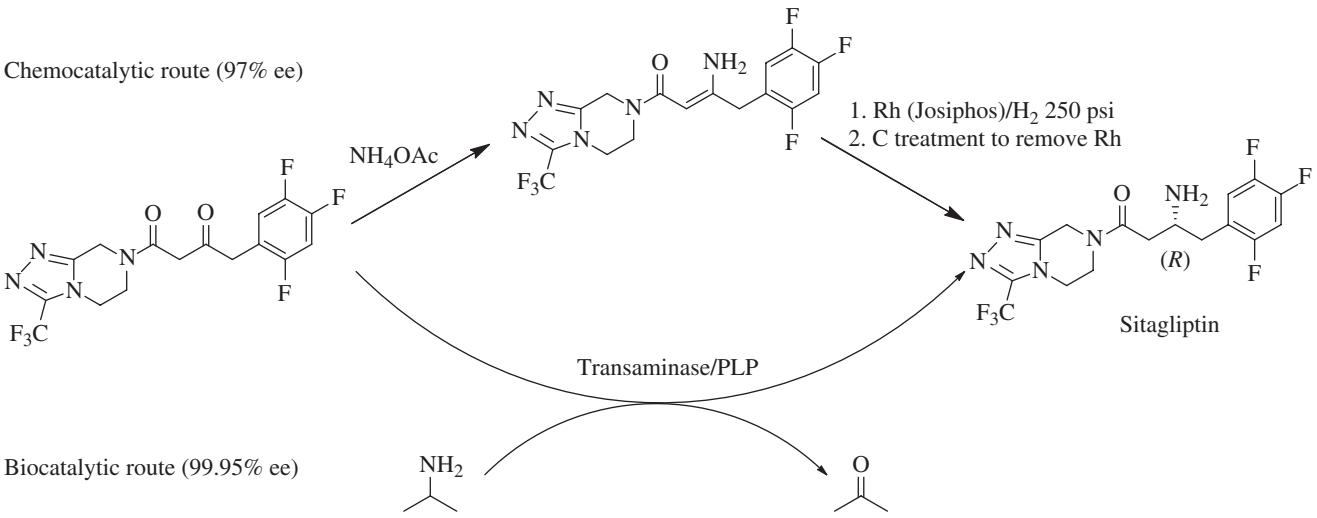

FIGURE 1.5
Two processes for sitagliptin.

ketone into the binding pocket of the enzyme. The amino residues surrounding the binding pocket were then engineered to provide the extra space leading to an enzyme with low activity, which was further improved up to a commercially viable level using *in vitro* evolution. Under optimized conditions, 6 g/l of the best variant in 50% aq. DMSO converted 200 g/l of the ketone substrate to sitagliptin of >99.95% ee with 92% yield. Compared with the rhodium-catalyzed asymmetric hydrogenation, the biocatalytic process displayed a 10–13% increase in overall yield and a 53% increase in productivity (kg/l/day). This resulted in a 19% reduction in total waste with the elimination of all heavy metals combined with a reduction in total manufacturing costs. Furthermore, the enzymatic reaction is run in multipurpose vessels, circumventing the need for specialized high-pressure hydrogenation equipment.

1.7.4 Biocatalytic Synthesis of the Fragrance Chemical (–) Ambrox (Ambrafuran)

The terpenoid molecule, (–)-8,12-epoxy-13,14,15,16-tetranorlabdane, so-called (–) ambrafuran or ambrox (a trade name of Firmenich SA), is one of the most valuable constituents of tincture of ambergris, a substance excreted by the sperm whale (*Physeter catodon* L.). It is much sought after by the perfumery industry and is produced commercially in a hemisynthesis from the diterpenic alcohol, sclareol [68]. The latter is readily extracted in sufficient quantities from clary sage (*Salvia sclarea* L.). The chemical synthesis (Figure 1.6) consists of seven steps involving long reaction times and hazardous reagents such as peracetic acid, lithium aluminum hydride, and butyl lithium; a stoichiometric oxidation with sodium periodate; and the generation of

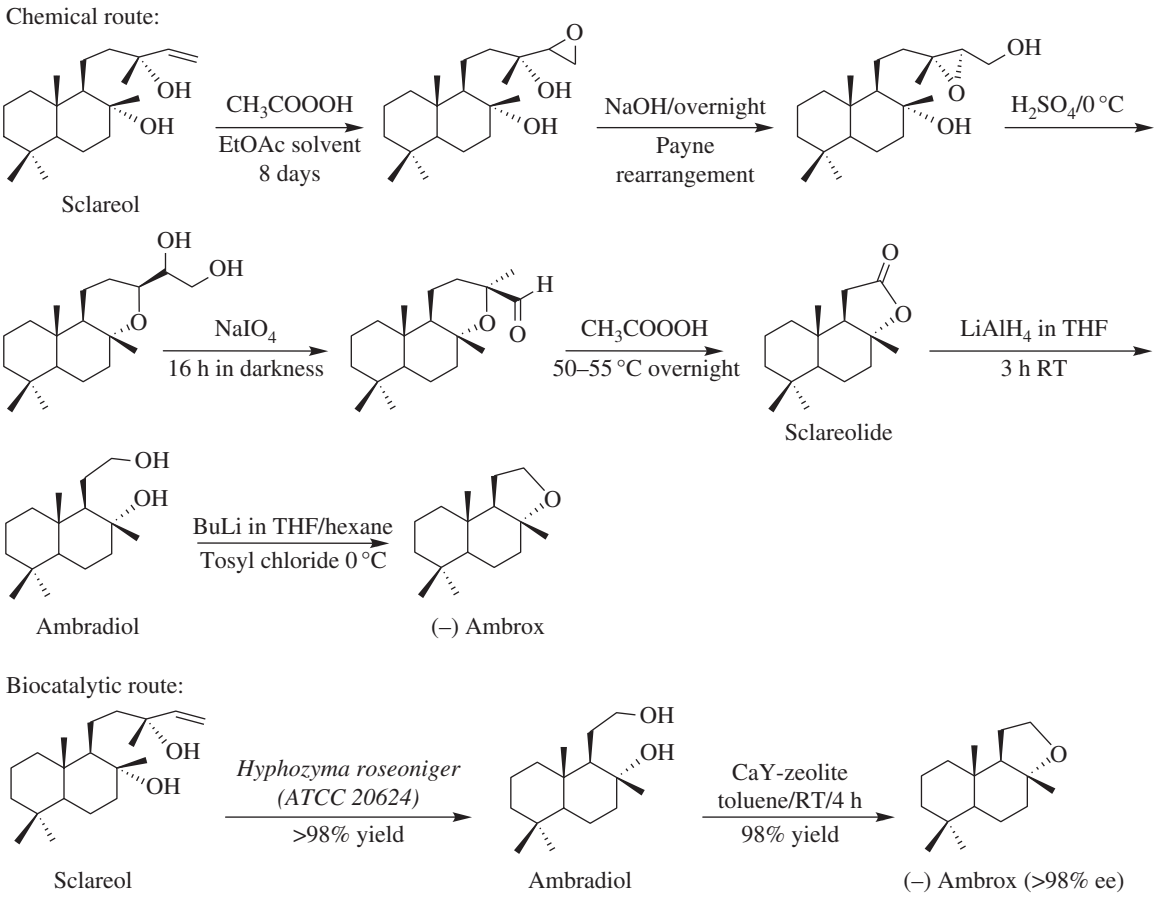

FIGURE 1.6
Two processes for (–) ambrafuran.

copious amounts of waste in addition to the 76% yield of the desired product. In stark contrast, a green, two-step process has been reported [69], which involves the conversion of sclareol to ambradiol as shown in Figure 1.6, catalyzed by whole cells of *Hyphozyma roseoniger*, followed by cyclization to (−) ambrafuran over a Ca-Y zeolite at ambient temperature, both steps proceeding in 98% yield.

1.8 CONCLUSIONS AND FUTURE PROSPECTS

Meaningful metrics for measuring greenness and sustainability are a *conditio sine qua non* for monitoring progress in the trend toward green manufacturing in the chemical and pharmaceutical industries. The widely accepted and complementary concepts of AE and E factors, together with an LCA, provide a sound basis for assessing the greenness and sustainability of different processes and products. Biocatalysis offers numerous benefits in this context. Reactions are conducted under mild conditions employing a catalyst that is biocompatible and biodegradable and derived from renewable resources, thus avoiding the scarcity and product contamination issues associated with the use of noble metal catalysts. Furthermore, processes are step economic and highly selective, resulting in higher product quality and reduced waste generation. In short, biocatalytic processes are green and sustainable, that is, they are more environmentally attractive and more cost-effective compared to classical chemical processes.

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