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Review

Evolution and current status of the phytochemistry of nitrogenous compounds

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Abstract

Nitrogen-containing and other secondary plant products have evolved as a consequence of the struggle between the plant and the animal kingdoms, the latter directly or indirectly thriving on plants. During evolution plants developed bioactive and exceedingly complicated chemical structures that serve the purpose of plant defense. It is this property of those plants that has been exploited by mankind as medicines, poisons and recreational drugs. Three classes of nitrogen-containing plant products are being reviewed in this article: the alkaloids, the cyanogenic glucosides/glucosinolates and the nonprotein amino acids. It is the interplay of different scientific disciplines such as chemistry, pharmacognosy, medicine, analytics, cell biology, molecular biology, botany and chemotaxonomy that form a new and exciting area called "phytochemistry". It is foreseeable that this integration of disciplines across traditional borders will bring new achievements in phytochemistry, as history has taught us already.

Keywords: Alkaloids; Cyanogenic glucosides/glucosinolates; Nonprotein amino acids; History; Discovery; Medicinal use; Cell cultures; *Raison d'être*; Biosynthesis

Contents

1. Introduction

The major classes of nitrogen containing "secondary metabolites" in the plant kingdom are the alkaloids, cyanogenic glucosides/glucosinolates and nonprotein amino

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acids. These different classes of nitrogenous compounds are all primarily derived from members of the 21 protein amino acids. Different levels of bioactivity have been demonstrated for most of the examples of these compounds discussed here. Selected alkaloids certainly have served for centuries as pharmaceuticals and today a number of alkaloids are used in medicine whether as such or after chemical modification. Cyanogenic glucosides and glucosinolates play a major role in the ecosystem as defense compounds. The nonprotein amino acids are classical antimetabolites inhibiting selected reactions in the primary metabolism of predators and herbivores. It seems that all of these compounds addressed in this review have a primary role in the ecosystem.

It is this interaction of nitrogen containing defense compounds produced by plants with foragers in the animal kingdom, which are directly or indirectly dependent on plants as a foodstuff, that makes one of the most fascinating complex interactions in our biosphere.

2. Alkaloids

2.1. Isolation, crystallization, structural determination of alkaloids

Alkaloids are the most mystical group of all phytochemicals. In ancient times these highly bioactive ingredients of plants have been used in all major human cultures. As examples, the latex of *Papaver somniferum* containing the active ingredients morphine and codeine is of Mediterranean origin. It has been used as a medicine against pain and cough for at least 5000 years. *Aconitum* species furnish the most poisonous plant alkaloid of the aconitine type in their root system. It was used as a spear-poison for whale hunting in the area of the Aleutian Islands and as a poison used for murder in ancient Rome to such an extent that growing the *Aconitum* plants as an ornamental was forbidden. A further use of specific plants was as psychedelic agents. Creams that were used by so-called ''witches'' contained different *Solanaceae* extracts (tropane alkaloids) dispersed in fat and caused dreams of flying if applied to the armpits. These compounds were also used during the Inquisition and by the Soviet Secret Service (KGB) to cause loss of will during interrogations. Largely, however, plants or plant extracts were used as medicines and the Greek/Roman antique knowledge expanded by the Arabian medicinal art was established by Ibn Sinai (called Avicenna, 980-1037) in his medicinal ''Qanun'' which was, after its translation from Arabic into Latin, the most comprehensive and influential book ever written on the therapeutic use of drug-plants. The Qanun's *materia medica* considers some 760 drugs, with comments on their application and effectiveness. Ibn Sinai recommended the testing of a new drug on animals and humans prior to general use! A huge picture of Avicenna was seen at the French Sorbonne until the French revolution. The use of Ibn Sinai's Qanun lasted for almost 1000 years. It was Paracelseus (1493–1541) who tried to reduce the Roman recipes, especially those by Galen and Andromachos which contained several dozen ingredients, to the actual active plant drug. This led to more simple prescriptions and a concentration on the principal active ingredients. In most cases these were – as we now know – alkaloids. It was a German pharmacist, Scheele (1742–1786) who isolated ingredients from plants in crystalline form such as tartaric acid, citric acid, oxalic acid and tannins. This coined the belief that plant-derived substances are principally acids. Scheele could be named the ''Founder of Phytochemistry''.

With the advent of the 19th century, we saw a more scientific approach towards pharmacognosy, the knowledge of drugs. The search for crystalline, pure plant products with bioactivity was in the hands of pharmacists, apothecaries. They maintained the knowledge from hundreds of years ago of the plant materials used in medicine. A firm knowledge existed of plant-derived drugs with defined action on the human body. It was these plants that were preferentially subjected to pharmaceutical investigations. The breakthrough occurred when the apothecary helper Sertürner of Paderborn at age 22 isolated crystals from opium that were very different from those previously isolated (which later turned out to be narcotine) by Derosne, a French pharmacist and competitor. Sertürner claimed that he had found the *principium opii*, which was not an acid, but rather a derivative of ammonia with alkaline properties that formed a salt. In 1817, Sertürner published a comprehensive paper in which he described in detail the isolation, crystallization, the microscopic crystal structure and the pharmacological properties, which he studied first in stray dogs and then in experiments on himself (Sertürner, 1817). He found that his pure crystals carried the sleep-generating properties of opium and he named this substance from *Papaver somniferum* morphium (Engl. Morphine), named after Morpheus, the god of sleep and the creator of dreams. The existence of plant-derived compounds with basic character caused an almost explosive international search for basic plant-derived compounds. Until the end of the century most of the major basic components from herbal drugs known at that time were isolated and crystallized. In 1819, Carl Meissner an apothecary from Halle, Germany, coined the name ''alkaloid'' for these types of alkaline nitrogen-containing compounds and the definition given by Winterstein and Trier (1910) still stands today: alkaloids are compounds with heterocyclic bound nitrogen atoms, with more or less expressed basic character, with pronounced physiological action, of complex molecular structure and are found in plants (and animals). After the discovery of morphine in (1805) 1817, the discoveries of narcotine (1817), xanthine (1817), strychnine (1818), piperine (1819), brucine (1819), quinine/quinidine (1820) and caffeine (1820) and many more followed in rapid sequence before the first amino acids, leucine and glycine, were discovered in 1820. It took another 84 years until all 20 protein amino acids were known.

Nine years after Sertürner's seminal discovery, the pharmacist and phytochemist Emanuel Merck initiated the commercial preparation of morphine which he sold to wholesale

companies that delivered this pain killer and antitussive to pharmacies. This was the cornerstone for the chemical factory Merck, Darmstadt, which was the first example connecting alkaloid research and industrial commerce. By the end of the 19th century, numerous pharmaceutical companies worldwide had opened their businesses and each one was either formed around alkaloids or had departments working with alkaloids. Alkaloids were a great commercial success. The physician did not have to rely on plant drugs from different origin with different potency, but he could use the pure, constant and unchanging material. This had an enormous impact on the medical profession.

Friedrich Rochleder (a student of von Liebig) at the University of Prague, was an excellent alkaloid chemist himself and Rochleder (1858) wrote a small, ninety-page contribution on the distribution of chemical constituents in the plant kingdom. He called it "Phytochemie". The same classification was undertaken by Hegnauer, slightly more than 100 years later – an enormous task! Our knowledge of chemical plant constituents had increased from 90 pages to 5725 pages (Hegnauer, 1962-1990).

However, the great challenge now existed as to the chemical structure of these alkaloids, characterized up to now by the pharmacognocists by crystal architecture, melting point, and formation of salts with different inorganic acids. Meanwhile, the chemists Lavosier, Gay-Lussac, Berzelius and von Liebig had invented and established elementary chemical analysis. Von Liebig analyzed morphine and obtained almost the correct sum formula of $C_{17}H_{19}NO_3$, missing only two hydrogen atoms. When it was seen that the chemistry of these plant bases was beyond the scope of pharmaceutical studies, the field of alkaloid research changed over to the hands of chemists. Numerous degradation methods for alkaloids were invented. Alkali melting and zinc dust distillation often revealed the heterocyclic nature of the alkaloid, whereas permanganate and silver acetate oxidation, saponification, ester and methoxy group cleavage, hydrolysis, complete methylation, etc. revealed, in some cases, the structure. The first degradation leading to the chemical structure of a heterocyclic base was performed on piperine (cited by Winterstein and Trier, 1910). In the 1860–1870's, synthesis of partial alkaloidal structures had already begun; coniine (the poison of *Conium maculatum*) was the first plant-specific alkaloid that was synthesized. Unlimited optimism swept the chemistry institutes and von Liebig wrote in his "Chemische Briefe" 1844, "There is experience enough that we hope that we will succeed to synthesize quinine and morphine ..." Twenty five years passed by, the prediction was not fulfilled and the Royal Prussian Academy of Sciences put forward a prize in 1870 for the solution of the problem to synthesize the plant alkaloids quinine, strychnine and morphine (Fig. 1). In vain! The timing was too early, the syntheses were attempted decades later, but the synthesis of quinine and morphine on a commercial scale and at a competitive price has not been achieved even today. Nevertheless, quinine was the most important alkaloid of the 19th century: structural elucidation and attempted syntheses made it by far the most researched alkaloid. Natural quinine isolated from *Cinchona* bark enabled Caucasian man to settle the tropics as quinine was a prophylactic against and a cure for malaria. Commercial synthesis was, therefore in high demand! We saw again during the Vietnam War (1963–1975) the importance of natural, tree-derived quinine compared to synthetic compounds such as chloroquine that resulted in resistance.

The synthesis of antipyrin by Knorr in 1883 at Erlangen demonstrated that new synthetic alkaloid-like compounds can have excellent therapeutic properties. This was a chance discovery of an "artificial" drug, chemically completely different from quinine, but acting as an antipyretic that was a commercial success. This discovery opened the synthetic pharmaceutical chemistry which no longer used plant products as targets or models. Finally the structure of cinchonine was solved and since quinine is the *p*-methoxy derivative of cinchonine, the structure of quinine was also settled. It was through the effort of the alkaloid chemists Königs, Skraup and Rabe that this problem was solved, over a period of 30 years from 1880 to 1910. The synthesis of quinine was finally formally achieved by the Woodward-Doering/Rabe-Kindler synthesis, a matter of utmost interesting and exciting controversy, recommended reading to every phytochemist (Seeman, 2007). Interestingly, the first stereoselective and unambiguous total synthesis of quinine was reported by Stork et al. in 2001, 181 years after the discovery of this alkaloid. Woodward also synthesized strychnine later in his career.

The study of alkaloids during the end of the 19th century brought 23 year old Perkin, Jr. from England to Munich to study degradation of isoquinoline alkaloids with ideas from Königs, in the stimulating department of Adolf von Bayer (indigo synthesis). He advanced within the period of three years to "Privat Dozent". Perkin, Jr. became one of the most successful alkaloid chemists after his return to Manchester, creating a prominent school of

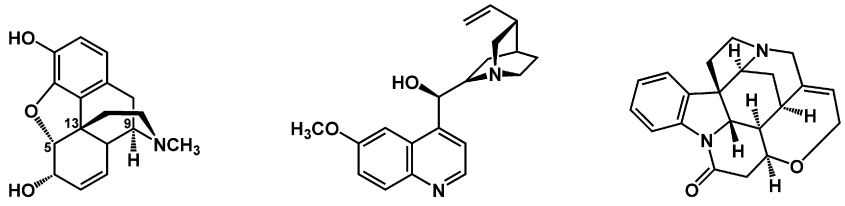

Fig. 1. Three alkaloids whose structure elucidation and/or chemical synthesis took 100 years or more.

organic chemistry there, and was joined in 1907 by the young Robert Robinson.

A second alkaloid that resisted structure clarification was morphine. It took ca. 500 scientific papers published over one and a half centuries during which time an enormous amount of detailed degradation- and synthesis-experiments went into the structural determination of morphine. At the end of the struggle with morphine's chemical structure elucidation, Gulland and Robinson (1924-1925) modified the existing proposed morphine (codeine) structure by shifting the bridge anchors from Carbon 5 to Carbon 13 (see Fig. 1). This proved finally to be the correct structure of morphine, even if not all stereochemical aspects were clarified. The final proof of the correctness of this structure came when Gates and Tschudi synthesized morphine in a 20 step procedure for the first time in Gates and Tschudi (1952,1955). The correctness of the morphine structure was confirmed again shortly after the total synthesis by one of the first structure elucidations/absolute configurations determined by X-ray crystallography (Mackay and Hodgkin, 1955). The time during both world wars and in between the wars showed no systematic search for new alkaloid classes, but rather a shift away from alkaloid research, while still exploring new methodologies for structure elucidation, perfection of synthetic methods and pharmacological testing of compounds for specific medical indications.

2.2. ''Raison d'être'' of alkaloids

Early on in the 19th century, pharmacists, chemists and botanists addressed the question as to why plants synthesize alkaloids. Why is it that a single plant species produces a specific alkaloid and none of the closely related species produce the same alkaloid? The philosopher Immanuel Kant (1724–1804) had stated: ''Nature never does anything superfluous and does not wastefully use resources not meant for specific purposes...Everything that Nature itself arranges has good reason''. The upcoming Darwinism in the middle of the 19th century may have sharpened this statement and Ernst Stahl (1888) in his famous book on ''Pflanzen und Schnecken'' formulated the opinion of that century on the ''*raison d'être*'' of secondary compounds by writing: ''So the great differences in the composition of the excretes and therewith also the metabolic processes will be placed closer to our understanding, if we look at these excretes as defense compounds which were gained in the struggle with the animal kingdom. The animal world had not only major impact on the morphology of the plant but also on their chemistry''. Furthermore, the great international authority in botany Pfeffer (1897) sums up: ''These, in some cases extremely poisonous metabolites, have obviously mainly ecological purposes, be it that they prevent grazing by animals or the intrusion by parasites''.

The new century, the 20th, saw a complete change in paradime with regards to this question, it was the chemists who brought about this change in thinking about the ''*raison d'être*'' of alkaloids. Interestingly it was started by a successful Swiss alkaloid chemist Amé Pictet, organic chemist of the University of Geneva, after whom the important Pictet-Spengler reaction was named. Between 1905 and 1907 Pictet formulated his ''fecal theory'' for the formation of alkaloids, which specifically suggested the disintegration of complex nitrogen-containing tissue components, as for instance protein, ''nucleins'' chlorophyll, etc. leads to relatively simple basic constituents. These products undergo subsequent increases in complexity by condensing with other compounds that exist side by side within the plant cell. Alkaloids are therefore excretion, waste and fecal products (Pictet, 1905; Pictet and Court, 1907). In a well documented experiment, Pictet and Chou (1916) tried to prove this possibility by heating caseine with HCl in the presence of formaldehyde (at that time the suspected reduction product of CO_2 in the process of photosynthesis) and slow addition of $CH_2(OCH_3)_2$ yielding, indeed, pyridine, dimethyl pyridine as well as desmethoxy salsolidine, an alkaloid! These ideas of Pictet were readily taken up by Robinson then 31 years of age and already for 5 years the first professor of pure and applied organic chemistry at the University of Sydney, who published an article on ''A theory of the mechanism of the phytochemical synthesis of certain alkaloids'' Robinson (1917a,1917b). He incubated succinic dialdehyde, methyl amine and acetone in dilute aqueous solution at room temperature for 30 min and got a small amount of tropinone (Fig. 2). Robinson wrote that this tropinone synthesis is ''...probably the method employed by the plant''. These ''biosyntheses'' were taken up again by Schöpf in the early 1930s under the title ''synthesis and transformations of natural products under physiological conditions''. He refined the Robinson tropinone synthesis by incubating succindialdehyde, methylamine and acetone dicarboxylic acid at 20 °C for three days and obtained tropinone in 90% yield (Schöpf, 1937). Schöpf concluded: ''the alkaloids appear as fortuitous reaction products of the cell, which are formed if corresponding precursors in a cell are originating side by side''. Robinson was a bit more careful and stated: ''the (above) conclusions have something to do, no doubt, with the mechanism of synthesis in the plant but they do not assist us to describe the detail of these mysterious operations''. The second half of the 20th century saw a come-back of the ideas of the 19th century, that secondary compounds, such as alkaloids, serve defense purposes against predators or act as pollinator attractants in the

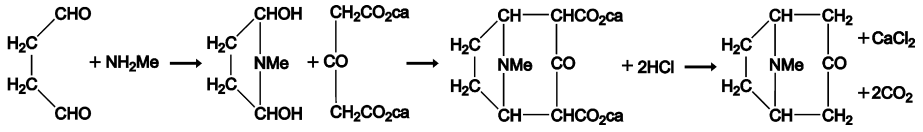

Fig. 2. Robert Robinson's tropinone synthesis (1917).

flower. A Max-Planck Institute was founded in the 1990s in Jena to clarify these important questions. This topic of the "*raison d'être*" will be dealt with in another contribution during this meeting.

Robinson's brilliant mind contributed greatly towards the arrangement of atoms within the molecules of morphine, strychnine, papaverine, nicotine, narcotine and many others. These discoveries led to Robinson's successful production of anti-malaria drugs based on the quinine structure. One of his achievements in total synthesis is the synthesis of tropinone. He has published a total of 718 papers. He was a consultant to many British companies and director of the Shell Chemical Company. Robert Robinson won the Nobel Prize in Chemistry 1947 "for his investigation on plant products of biological importance especially the alkaloids". Alkaloid research was worthy of a Nobel Prize and this fact gave an enormous boost to both academic and industrial research.

R.H.F. Manske, a former coworker of Sir Robert Robinson decided in 1949/1950 to compile the knowledge of alkaloids of that time and to bring order to this discipline by planning and publishing "The Alkaloids" (Academic Press) in 5 volumes in the form of a "Handbuch". Manske wrote in the preface of Vol. 1 "Alkaloids occupy an important position in commerce and forensic chemistry, and in medicine they play a role that, in many instances, may be classed as indispensable. Because of their multiplicity of type and their unique and manifold reactions, alkaloids offer to the chemist a challenge shared by no other single class of organic compounds." After the five volumes and two supplement volumes were published, Manske wrote "The explosive advance in the chemistry of the indole alkaloids in recent years has been occasioned not only by their intrinsic interest as problems in chemistry but by the possibility that some at least might have therapeutic value." (Preface to Vol. VIII). "The Alkaloids" series continued and is now in the 60th year and 63rd volume. Manske's hope and expectation to find compounds of therapeutic value was soon fulfilled.

2.3. Activity guided fractionation of plant extracts

After the Second World War, European industry was of the opinion that organic chemistry had many more scientific problems in store, but it was not likely that products of great economic importance would be found in the class of alkaloids anymore. How wrong this opinion was! Already in 1952 reserpine was discovered in the CIBA Pharmaceutical Company at Basel. It was an indole alkaloid. This class of alkaloid was known many decades earlier, but the isolated compounds such as yohimbine, harmaline, strychnine, physostigmine, *Evodia* alkaloids and others found only little interest and application in the medical community. Two years after its discovery, reserpine was introduced on the market as an antihypertensive drug and antipsychotic drug. Reserpine was a very successful drug, but because of a narrow therapeutic index and some side effects, it was abandoned. Other indole alkaloids were ajmaline, which acts on the heart muscle, blocks local sodium channels and is a class I antiarrhythmic agent. Raubasine (ajmalicine) can be obtained by hydrogenation of serpentine which is a side product of the reserpine isolation process from different *Rauvolfia* species. Ajmalicine is used as an antihypertonic, sedative and tranquilizer. It also increases the supply of oxygen to cerebral tissues and is beneficial to post-stroke rehabilitation. The plant genus *Rauvolfia* proved to be an excellent source of indole alkaloids that were extensively used for about two decades, before they were largely overtaken by superior synthetic products. While the European pharmaceutical companies had concentrated on the *Rauvolfia* indole alkaloids acting on heart and circulatory diseases, the US-based company Eli-Lilly and the Cancer Research Center, Canada were independently interested in a pantropic plant, *Catharanthus roseus* used in traditional medicine in Jamaica to combat diabetes. All attempts to influence the blood sugar level in animals with various *Catharanthus* extracts conducted by both institutions were uniformly negative. However, the Canadian group found that injection of solutions of a crystalline indole alkaloid isolated from that plant into rats depressed the white blood cell count also in bone marrow. That compound was named vincaleucoblastine (=vinblastine, VLB) (Noble et al., 1958). The Eli-Lilly group on the other side, headed by Gordon H. Svoboda (Johnson et al., 1959; Svoboda et al., 1962), interested by the claimed anti-diabetes properties of that plant, independently discovered the oncolytic activity of

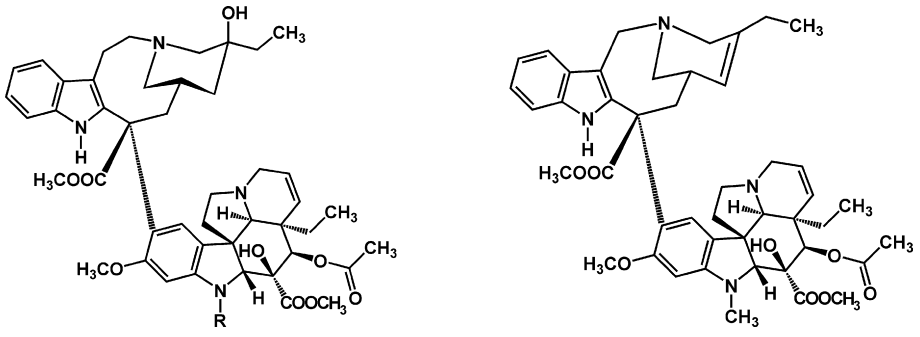

Fig. 3. Natural and semisynthetic dimeric indole alkaloids, chemotherapeutic agents against a variety of human neoplasms.

VLB and also of a second alkaloid, vincristine (VCR) (Fig. 3). Both were oncolytic agents representing new and hitherto unknown dimeric alkaloids that were effective chemotherapeutic agents directed against a variety of human neoplasms. The occurrence of these two dimeric alkaloids in the leaves of the *C. roseus* plant is very low (0.001–0.002% dwt). The extraction is costly and tedious. At the time, these two alkaloids were the most expensive medically applied compounds, with a price of more than $100,000 per gram at the pharmacy level. With time, however, semi-synthetic processes for VLB and VCR have been worked out starting with the much more available monomers vindoline and catharanthine. This discovery had an enormous impact on natural product chemistry, on phytochemistry and related disciplines worldwide. Alkaloids were recognized to inhibit, in some cases even cure, cancerous growth in human patients. Phytochemistry gained enormous respect; alkaloids of plant origin took again a triumphant march through natural sciences and medicine. Volume 27 of Lloydia (1964) the Journal of Pharmacognosy based on the fifth annual meeting of the American Society of Pharmacognosy at Pittsburgh, PA, was entirely devoted to "Chemistry and Biological Activities of *Catharanthus*, *Vinca* and Related Indole Alkaloids". Enthusiasm prevailed. All modern methodologies have been involved in *Catharanthus* research and discussed at Pittsburgh. Biemann and the two Spitellers, then as postdocs, used a mass spectrometer which was sitting idle at MIT and fortunately chose an alkaloid-rich plant (*Aspidosperma quebracho – blanco*) as one of the first MS investigations. The alkaloids showed clear and distinct fragmentation patterns for structure elucidation which strongly enhanced the demand for these mass spectrometers "which yield these wonder methods" for structure elucidation of alkaloids (Biemann et al., 1961, 1963). With the knowledge of the *Aspidosperma* alkaloid carbon skeleton, the mass spectrometric methodology solved an unaccountable number of structures of *Catharanthus* and other alkaloids. Plant cell cultures were established with the hope to produce *Catharanthus* alkaloids in higher yield and independent of agriculture. The newly established NMR techniques were used for structure elucidation of dimeric alkaloids. The *Catharanthus* alkaloids were evaluated by thin layer chromatography and the use of ceric ammonium sulfate as spray reagent proved useful to distinguish structural types. The selective extraction and gradient pH extraction techniques for those indole alkaloids were invented. The chemical synthesis of selected *Vinca* alkaloids was started. The chemotaxonomic considerations in the Apocynaceae were published and all members of the *Catharanthus* genus from Madagascar were carefully checked as to their phytochemical composition. Several then recently established cancerous human cell lines were tested with regard to their response towards these alkaloids. Tritiated vinblastine was injected into rats and its metabolism was studied. Chemical modification of these highly physiologically active molecules yielded structure-activity relationships. Microbial transformations of VLB were attempted to obtain new molecules with different biological activities. The Pittsburgh meeting was the first and most comprehensive phytochemical meeting of its time and served as a model for future meetings.

The dimeric indole alkaloids VBL and VCR received fast approval by the American Medical Association (now FDA) and both drugs have been commercially available now for more than 40 years, having prolonged or saved the lives of countless cancer patients.

There is still room, however, for improvement of these dimeric alkaloids, as has been shown recently by the synthesis and market introduction of navelbine (Potier, 1989), a semisynthetic derivative of anhydrovinblastine in which the indole-ethylamine side-chain is shortened by one carbon and a double bond is introduced (Fig. 3). This compound is orally active against bronchial carcinoma and others, has less toxic side effects, and as all the other commercial dimeric indole alkaloids, prevents the polymerization of tubulin into microtubules and thus inhibits mitosis of cancerous cells. Navelbine sales are presently $0.6 billion and rising.

Up to now, all the alkaloids that proved to be useful in human therapy occurred in plants that enjoyed a folklore reputation, right or wrong. Monroe Wall of the USDA was (during WWII and then post-war) instructed to screen plant samples first for the occurrence of alternative rubber, then for phytosteroids. In the late 1950s, the national cancer institute (NCI) launched a program to screen plants for anticancer activity. Wall was convinced by the NCI to send the first thousand frozen plant extracts, which were still kept in storage, so that NCI could perform antitumor

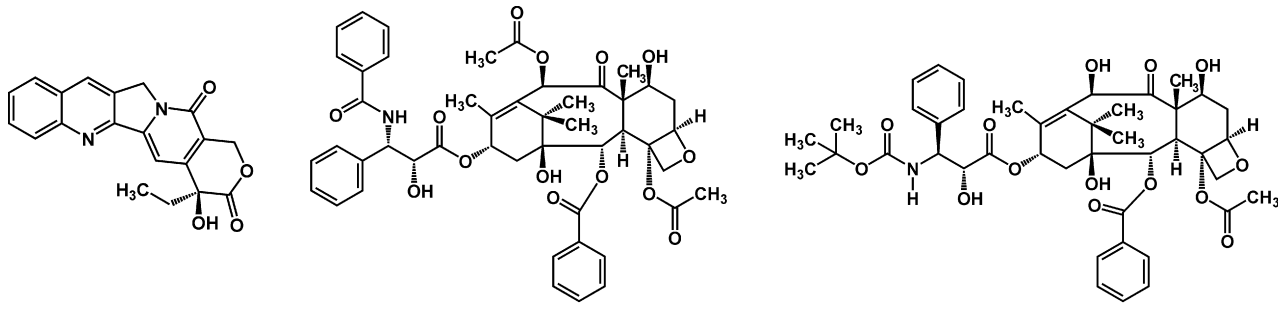

Fig. 4. Major taxans used in tumor therapy.

activity testing. One plant extract from *Camptotheca acuminata* demonstrated potent cytotoxic activity against tumor cell lines. The USDA, however, did not share Monroe Wall's enthusiasm for anticancer plant research. In 1960 Wall and, two years later, the organic chemist Wani (originally from Bombay) moved to the Research Triangle Institute in North Carolina with a promise of funding from the NCI. The natural product laboratory was successful and the compound from *Camptotheca* was isolated in crystalline form using bioactivity-guided fractionation (Fig. 4). Its structure was deciphered by X-ray methodology and proved to be an alkaloid which was named "camptothecin" (Wall et al., 1966). In spite of its strong cytotoxic action the application of this alkaloid to patients proved to be difficult because of its insolubility in water. The water soluble sodium salt gave positive results. Camptothecin languished for ca. 15 years until its unique mode of action by inhibiting the enzyme topoisomerase I was discovered. The DNA replication of cancer cells was inactivated by binding to and stabilizing the covalent DNA-topoisomerase complex. This finding brought the water soluble derivatives topotecan and irinotecan, produced by total synthesis, to clinical use. These compounds are active against ovarian, breast and colon cancers. New derivatives originating from the natural alkaloid are presently being pursued.

Wall and Wani at the Research Triangle Institute decided in 1964, parallel to their work on *Camptotheca,* to pursue isolation of the cytotoxic ingredient of *Taxus brevifolia* bark extract, which had been noticed by other groups, but who declined to continue to work on it because of its high cytotoxicity that might be indicative of a poor drug candidate. They followed again their bioassay-directed fractionation protocols and obtained a crystalline substance. Due to the presence of hydroxyl groups in the molecule, they named the compound "taxol". Structure elucidation proved extremely difficult, but after mass spectrometry, X-ray crystallography and NMR spectroscopy, the structure was finally solved (Wani et al., 1971). The compound turned out to be a pseudoalkaloid, where the nitrogen atom was not heterocyclic bonded (Fig. 4). The interest in this substance increased greatly in 1979 when it turned out that the mechanism of action of taxol was unique; taxol acts as a spindle poison during cell division by binding to microtubules, causing assembly of tubulin to microtubules and stabilizing them against depolymerization. The shortage of *Taxus brevifolia* bark was overcome when a rich source of 10-deacetylbaccatine was found in the needles of the European *Taxus baccata* which in turn could be converted with the synthetic aromatic side chains to semisynthetic taxol or the more water soluble taxotere (Fig. 4). Both the semisynthesis of taxol from a renewable plant source and the modification of the taxol structure to yield taxotere were directed by Pierre Potier at Gif-sur-Yvette (Guenard et al., 1993). Both taxol and taxotere are now blockbusters on the pharmaceutical market. Recently taxotere, with sales over two billion dollars per year and still rising, has definitively overtaken the sales of taxol ($1.2 billion). The use of alkaloids and their derivatives continues to be a success story in the medical treatment of mankind.

2.4. The biosynthesis of plant alkaloids

In their book "Die Alkaloide", Winterstein and Trier in 1910 included, with great foresight, a chapter on the formation of alkaloids. At that time, they had already formulated five principle mechanisms for the biosynthesis of these bases: the methylation of imines, amines and amino acids, the methylation of phenols, the condensation of phenols and alcohols, the condensation of alkamines (oxyamino acids, etc.) and the condensation with "formic acid". They predicted that 3,4-dihydroxylated phenylethylamine could condense with a 3,4-dihydroxylated phenylacetaldehyde with a loss of water to form the first alkaloid, what we call today norlaudanosoline (=tetrahydropapaveroline), which in turn could be the precursor of more than 2000 isoquinoline bases. Thirty five years later, theoretical schemes were put forward for the biosynthesis of more sophisticated alkaloids (e.g. Woodward, 1948) involving the oxidative fission of the catechol ring, later called "Woodward fission" leading to intermediates that should explain the formation of the alkaloids strychnine, serpentine, ajmaline, quinine, emetine and other structural types. In a note attached to this paper, the Nobel laureate Robinson wrote: "It is obvious that by chopping up the benzene ring, recombining the fragments, almost any kind of structure can be obtained". The Woodward fission stimulated biosynthetic speculations, but proved to be incorrect. The then unknown natural product secologanin (Brechbühler-Bader et al., 1968; Loew et al., 1968; Battersby et al., 1968,1969) was the true partner for the Pictet–Spengler reaction with

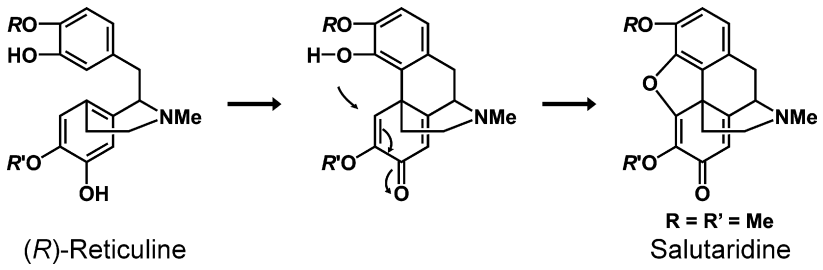

Fig. 5. On theoretical grounds, predicted biogenetic path from reticuline to salutaridine by radical pairing, the key step in morphine formation in *Papaver*. (Barton and Cohen, 1957).

tryptamine and also dopamine. A further groundbreaking hypothesis was formulated by Barton and Cohen – the oxidation of phenols by one-electron transfer that afford phenolic radicals which, by radical pairing, form new C–C and/or C–O bonds either intra- or intermolecularly (Barton and Cohen, 1957). This proposal explained the formation of certain plant alkaloids (especially the formation of salutaridine from (*R*)-reticuline) (Fig. 5). The biocatalysts involved were found and turned out to involve cytochrome P450 (Zenk et al., 1989), as shown again 30 years later. Extremely helpful books for alkaloid researchers appeared (e.g. Cordell, 1981; Dewick, 1997) that provided guidance for chemists, pharmacists, biologists and later for molecular biologist – let us say generally for phytochemists.

The great success of biosynthetic experimentation came with the introduction of isotopes, especially of ^{14}C position-specific putative precursors, allowing the living plant to metabolize the label and form the compounds in question. Labeling, *per se*, however, did not prove precursorship, rather the target compound had to be isolated, rigorously purified and subsequently degraded by tedious chemical means to prove direct precursor-product relationship. The entire biosynthetic experimentation and results regarding alkaloids were published in a magnificent chapter by Spenser (Spenser, 1968) containing 726 references. This survey showed that the initial phase in the investigation of the biochemistry of alkaloids was at that time almost completed. Spenser pointed out "it is unlikely that a great deal of further information emerges by this experimental approach" and he further stated that "the time is ripe for an attack on the enzymology and kinetics of the routes to secondary biosynthesis". This was the signal heard and felt by everyone working in the field. Interestingly, this was the first time that alkaloid research was not directly linked to any application. Alkaloid biosynthesis was *l'art pour l'art*; one was curious how nature was forming these complex molecules, finding the enzymes, the intermediates and the compartmentalization. (Absolutely nobody at that time was envisioning transgenic plants! It only took a bit more than 10 years for such plants to become a reality.) It turned out that higher plants were difficult to work with. They had a sluggish rate of expression of secondary metabolites often spread over an entire vegetation period, coupled to low stationary levels of corresponding biosynthetic enzymes and the large amount of acids, tannins and other phenolics in their vacuoles that interferred with the extraction of active enzymes from plant tissue. A tremendous encouragement and stimulation of ambition was given by Neish (1961) by the early discovery of tyrosine ammonia lyase, which was published in the very first volume of "Phytochemistry" on page 1, the first ever published article there, and by Koukol and Conn (1961) on phenylalanine ammonium lyase, both fundamental enzymes of secondary metabolism and of lignin. In order to overcome the above-listed problems with differentiated plants, we established in 1970 plant suspension cultures from different plant species to provide a source of enzymes of secondary metabolism. The success of this methodology has been reported previously as part of the celebration of the first thirty years of our journal "Phytochemistry" under the title requested by then editor Harborne "Chasing the enzymes of secondary metabolism: Plant cell cultures as a pot of gold" (Zenk, 1991). This success story still continues; for the last five years, the pseudoalkaloid taxol (generic name paclitaxel) has been produced by Phyton Biotech through commercial fermentation of *Taxus* cells for Bristol-Myers-Squibb in a large scale cGMP fermentation facil-

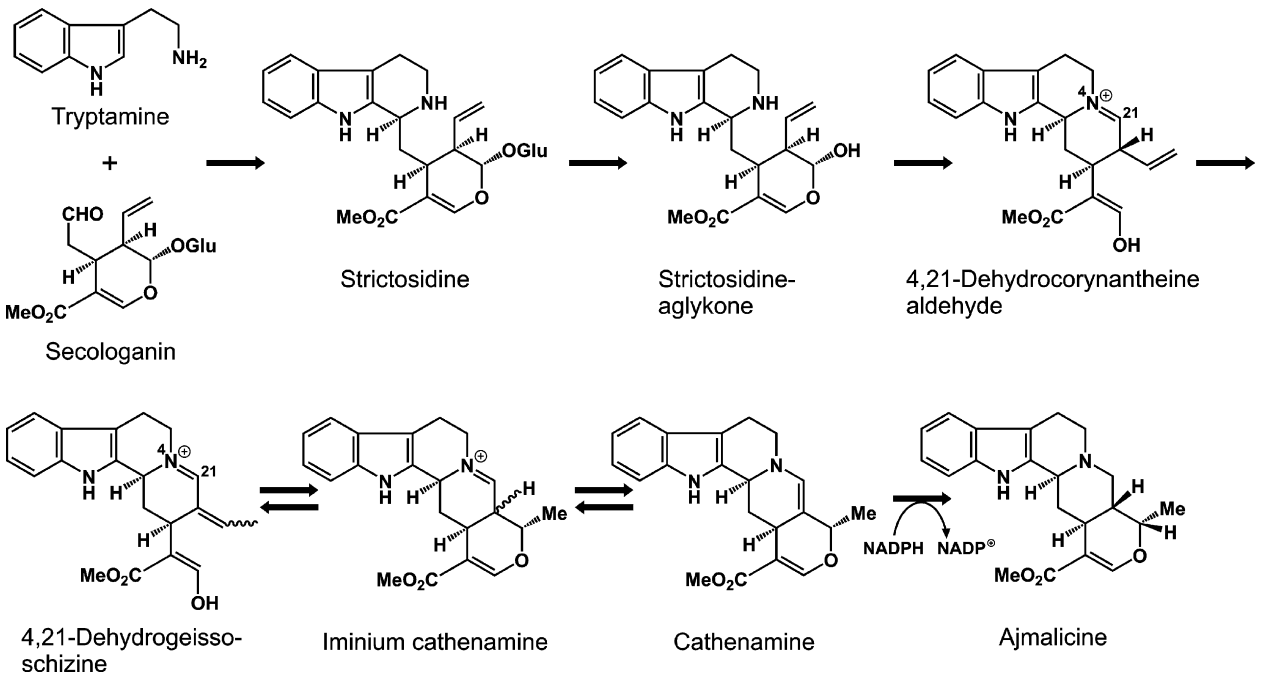

Fig. 6. The first complete enzymatic formation of an alkaloid: ajmalicine (=raubasine).

ity in Hamburg, Germany. If this extraordinarily complex molecule can be produced on a commercial scale, one can be optimistic about producing just about any given plant-derived secondary compound by cell culture technology, if time and financial resources allow. A strategy to reach this goal that we reported early on should prove useful (Zenk et al., 1977). A selected *Catharanthus roseus* cell culture strain was developed that produced monoterpenoid indole alkaloids in yields higher that the differentiated plant from which the cell culture was derived. This cell suspension culture was the source of enzymes for the cell-free biosynthesis of the target alkaloids. We worked out the entire pathway from tryptamine and secologanin to ajmalicine (=raubasine) at the enzyme level which was used at that time commercially (Zenk, 1980). It was the first complete enzymatic synthesis of an alkaloid (Fig. 6).

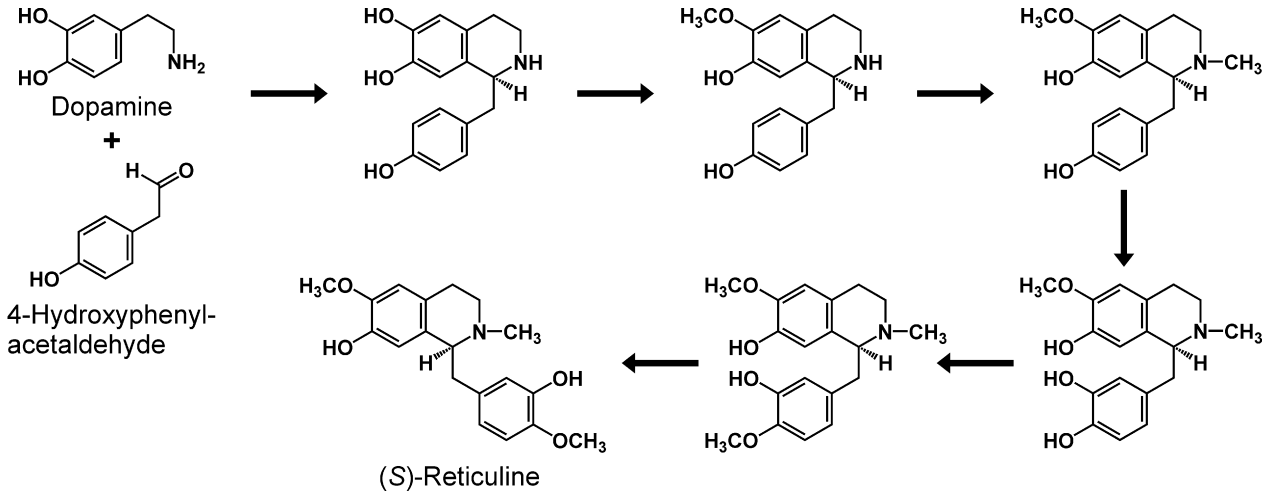

Fig. 7. The pathway from dopamine and 4-hydroxyphenylacetaldehyde to (*S*)-reticuline, the precursor of numerous benzylisoquinoline alkaloids.

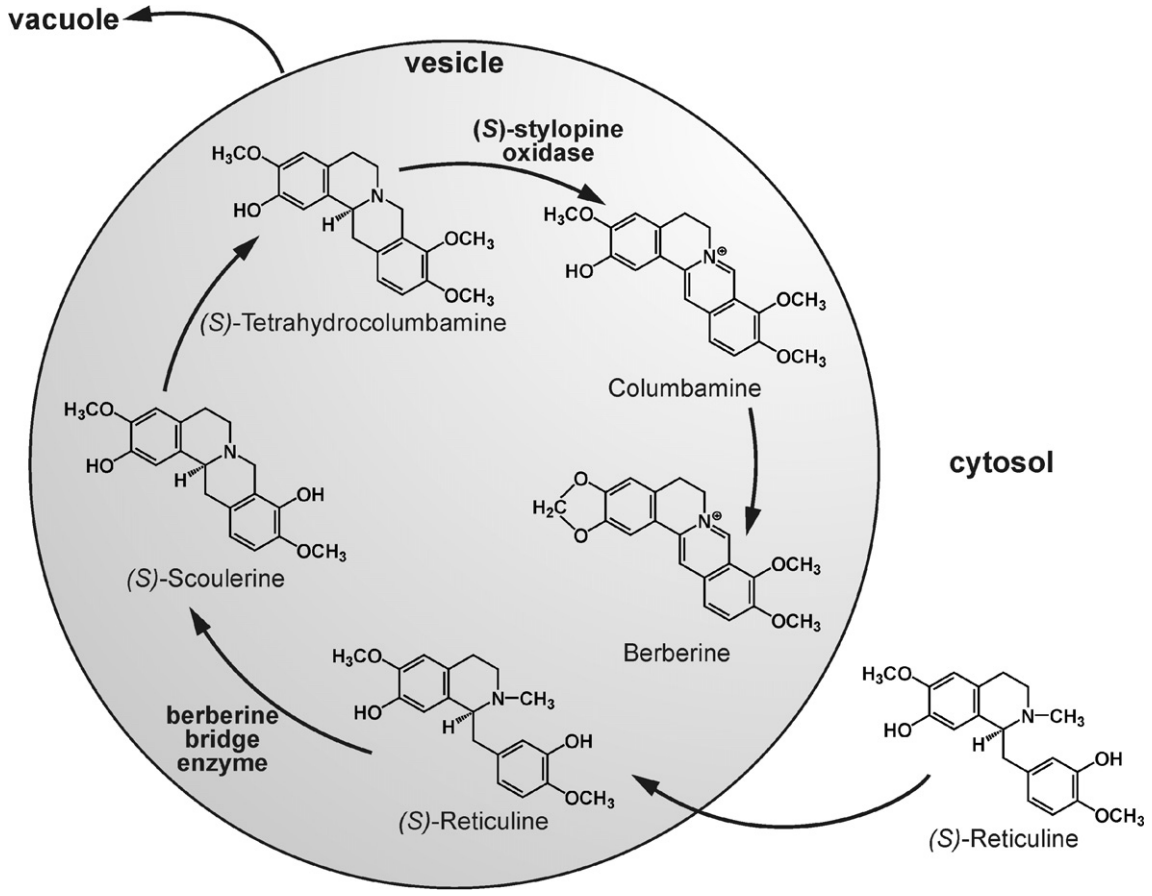

Fig. 8. The berberine biosynthetic pathway is localized in a vesicle containing all enzymes for the formation of this alkaloid. The vesicles fuse with the tonoplast membrane and release their alkaloids into the central vacuole.

The entrance reaction to these indole alkaloids was catalyzed by an enzyme that condensed tryptamine and secologanin in a Pictet-Spengler type reaction yielding 3α(*S*)-strictosidine (Stöckigt and Zenk, 1977) and not as previously claimed the 3β(*R*) epimer vincoside. Strictosidine synthase (Treimer and Zenk, 1979) catalyzes the entrance reaction to the multitude of monoterpenoid indole alkaloids. The enzyme from *Rauvolfia* (Hampp and Zenk, 1988) was partly sequenced, cloned and functionally expressed in *E. coli* (Kutchan, 1989). This was the first alkaloid cDNA isolated and functionally expressed in a heterologous system. It was already found at that time (Treimer and Zenk, 1979) that this enzyme accepts also 7-methyl, 6-hydroxy, 7-fluoro, 5-fluoro and 5-hydroxy tryptamines, yielding the corresponding substituted strictosidines. This line of research has been taken up again recently (McCoy and O'Connor, 2006) to produce "unnatural" indole alkaloids.

It was a lucky circumstance that more than 40 species of the genus *Berberis* cultivated in our laboratory in Munich were able to produce in the cell suspension stage up to 1.7 g/l medium berberine-type alkaloids with a cell mass of ca. 15 g dwt/l (Hinz and Zenk, 1981). With these cell cultures in hand, we were able next to study the enzymatic synthesis of berberine, a member of the large group of tetrahydrobenzylisoquinoline alkaloids in the plant kingdom. The central intermediate of this pathway is the Pictet-Spengler type stereoselective condensation product between dopamine and *p*-hydroxyphenylacetaldehyde, the trihydroxylated (*S*)-norcoclaurine and not, as Winterstein and Trier, 1910 envisioned, the tetraoxygenated (*S*)-norlaudanosoline, which many alkaloid researchers also assumed in the 1950's to 1980's. Surprisingly, the methyltransferases involved in the (*S*)-reticuline pathway demonstrated relaxed substrate specificity such that the correct trihydroxylated but also the incorrect tetrahydroxylated substrates could be methylated both *in vivo* and *in vitro*. Interestingly, there is a scientific controversy as to the protein structure and function of (*S*)-norcoclaurine synthase (EC 4.2.1.78). One norcoclaurine synthase has been isolated and partly characterized from cell suspension cultures of *Thalictrum flavum* ssp. *glaucum* and its cDNA isolated (Samanani et al., 2004). The protein seems to be a pathogenesis-related enzyme. A second recently isolated cDNA from *Coptis japonica* also catalyzes the norcoclaurine synthase reaction. It is related to 2-oxoglutarate-dependent dioxygenases, was fully characterized and shown to catalyze formation of the (*S*)-configured alkaloidal precursor (Minami et al., 2007). It seems unlikely that two completely unrelated enzymes will catalyze one and the same stereoselective reaction.

We could further on conclusively demonstrate that the pathway from (*S*)-norcoclaurine proceeds stepwise by three methylation reactions (Stadler and Zenk, 1990) and one hydroxylation (Pauli and Kutchan, 1998) to the central and branch point intermediate (*S*)-reticuline (Fig. 7). The pathway leading from (*S*)-reticuline to berberine is depicted in Fig. 8; four enzymes are involved. The cDNAs that encode these enzymes were isolated and functionally expressed by two groups, from *Berberis* (Dittrich and Kutchan, 1991) and from *Coptis* (Ikezawa et al., 2003). At least two of these enzymes were housed in Golgi-derived particles with the density of $\delta = 1.14$ g/cm^3 in a sucrose density gradient. These vesicles have been found in members of four different plant families (*Annonaceae*, *Berberidaceae*, *Ranunculaceae*, *Menispermaceae*) as well as in differentiated plants. Using post-embedding immunogold techniques, the cytological localization of two enzymes of isoquinoline biosynthesis, the berberine bridge enzyme and the (*S*)-tetrahydroprotoberberine oxidase, was demonstrated. Electron-microscopic examination revealed their exclusive compartmentalization within these endoplasmic reticulum-derived vesicles. After these vesicles have fused with the central vacuole, they release their alkaloid contents into this compartment (Bock et al., 2002) (Fig. 8).

Tropane alkaloids have been known for ages to yield powerful bioactive compounds. They are derived from

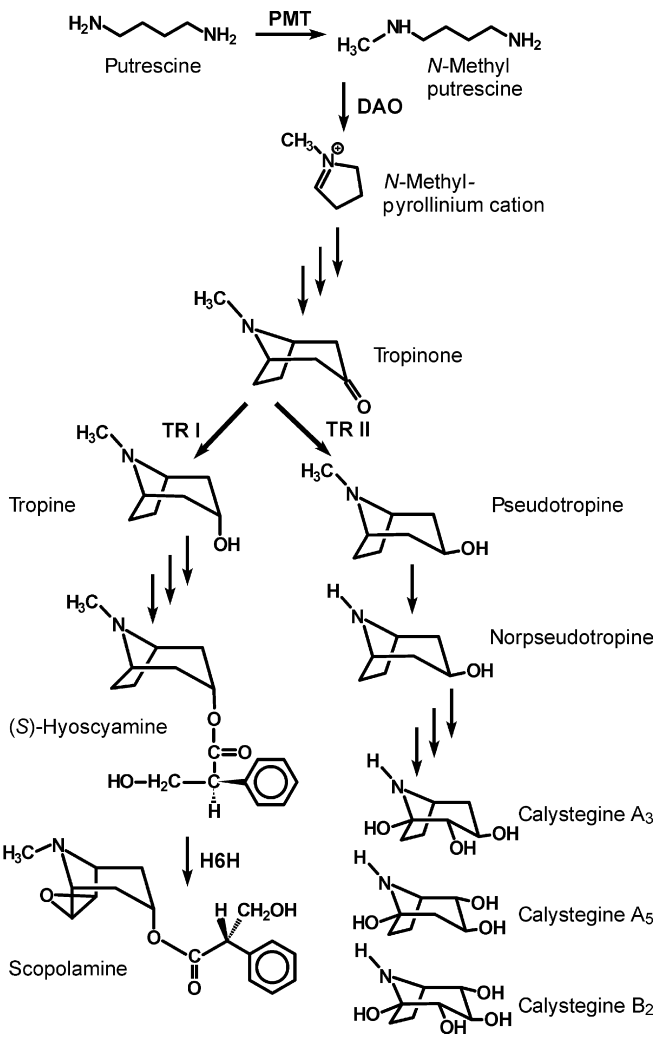

Fig. 9. The biosynthesis of select tropine and pseudotropine alkaloids (Dräger, 2004).

putrescine via an important branch point intermediate tropinone which can be reduced steroselectively either by tropinone reductase I leading to the scopolamine branch or by tropinone reductase II leading to the calystegine branch (e.g. Dräger, 2004) (Fig. 9). The bifunctional hyoscyamine 6-hydroxylase (H6H) is a 2-oxoglutarate-dependent dioxygenase that catalyzes the oxidative reactions in the biosynthetic pathway from hyoscyamine to scopolamine, an esteemed anticholinergic alkaloid of the *Solanaceae*. The availability of the H6H gene made it possible to transform *Atropa belladonna*, rich in hyoscyamine, into a plant that now contains almost exclusively scopolamine (Yun et al., 1992). The pathway catalyzed by tropinone reductase II leading to the calystegines yields compounds that are glucosidase inhibitors, but are suspected of further yet unknown functions (Biastoff and Dräger, 2007). The tropane alkaloids and their semisynthetic derivatives are most valuable in medicine; scopolamine against motion sickness, scopolamine-*N*-butylbromide against intestinal spasms, ipratropinium bromide (Atrovent) against asthma and the novel tiotropinumbromide (Spriva) to treat chronic obstructive pulmonary disease.

The pyrrolizidine alkaloids for which ample information is available will be dealt with in this series of lectures by Thomas Hartmann.

In an extraordinarily original and experimentally difficult study, Stöckigt (Mainz, Germany) worked out the complete pathway of another indole alkaloid, ajmaline. This alkaloid is still used as an antiarrhythmic drug, derived from *Rauvolfia serpentina*. Over a period of more than two decades, the complete pathway leading to ajmaline (and some side alkaloids) involving 10 enzymes was unraveled (Fig. 10). These enzymes were discovered, purified and characterized (summarized in Ruppert et al., 2005). Half of these enzymes were sequenced, cloned and heterologously expressed. In an excellent string of papers, four proteins of the ajmaline pathway were crystallized and the X-ray structures were described at 2.6-Å resolution (Stöckigt et al., 2007). It is the ambition to characterize by X-ray diffraction, for the first time ever, all individual enzymes involved in an entire alkaloidal pathway. For each enzyme, this will allow a 3D view of the catalytic center. The 3D structures have to be corroborated by site-directed mutagensis and crystallization/structural determination of enzyme-substrate complexes. With the combined knowledge of the enzyme and enzyme-substrate complex, a rational enzyme design will be possible in the future. The first experiments conducted with strictosidine synthase show that by relaxing the strictosidine synthase substrate specificity this vision can be realized (McCoy and O'Connor, 2006). Transformation of plants with genes carrying new catalytic activities can play a major role in future agriculture to produce designed fine chemicals at this time of dwindling petroleum reserves – certainly a future goal of phytochemistry.

The "Holy Grail" of alkaloid biosynthesis was to decipher the pathway leading to morphine, the intriguingly complex alkaloid whose structural clarification took over

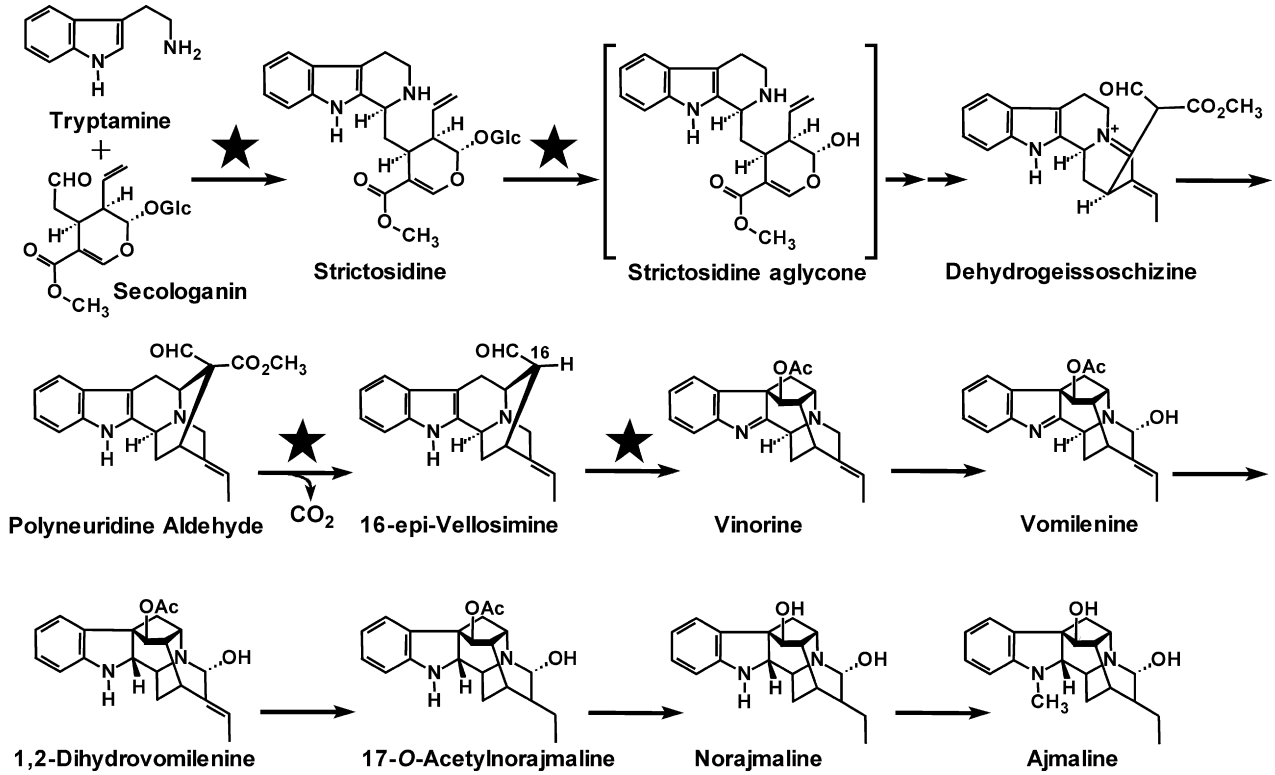

Fig. 10. The enzymatic formation of ajmaline and the crystallization (stars) of recombinant enzymes. (Ruppert et al., 2005).

100 years and the first chemical synthesis 150 years. Morphine was first studied at the precursor feeding level, then at the level of the individual enzymes and lastly at the gene level. It was a truly international effort. The precursor feeding experiments up to 1968 and a rough proposal of the pathway involved were summarized (Kirby, 1967; Spenser, 1968) and involved mainly the groups of D.H.R. Barton (London, UK), Battersby (Cambridge, UK), Brochmann-Hanson (San Francisco, USA), Rapoport (Berkeley, USA). The pathway as it now stands, after additional precursor feeding experiments and enzymological clarification of the individual steps as worked out by the group in Munich/Halle, is shown in this review starting from (*S*)-reticuline in Fig. 11. The pathway to morphine comprises a total of 19 chemical steps which lead from 2 molecules of l-tyrosine to the highly condensed molecule of morphine containing 5 asymmetric centers. Only two steps, the vinyl ether cleavage enzyme and the codeine demethylation steps in the poppy plant are still unclear. All other enzymes, in selected cases purified to homogeneity, are in hand and nine individual genes of the morphine pathway are cloned in the Kutchan laboratory (Ziegler et al., 2006 and references contained therein). Two genes of the morphine pathway, one coding for an early enzyme (CYP80B) and a second one coding for a late enzyme (codeine reductase) were reintroduced into the poppy plant, expressed and the yields of morphine analyzed. The first gene expressed under greenhouse conditions yielded up to 4.5-fold more morphine and the second 20% improvement of morphine compared to the wild type plant under field trials (Frick et al., 2007; Larkin et al., 2007). A breakthrough was achieved in conventional poppy breeding when Tony Fist at Tasmanian Alkaloid Ltd. and his associates obtained, by chemical mutagenesis of seeds, a plant that accumulated about 3% of thebaine and small amounts of oripavine instead of morphine and codeine (Milgate et al., 2004). This mutant named "top1" has been successfully deployed as the long sought agricultural supply of thebaine, which serves as the starter molecule for the synthesis of modern analgesic and competitive antagonists at the opioid receptor level.

3. Cyanogenic Glucosides/Glucosinolates

A second class of nitrogen-containing compounds includes the cyanogenic glucosides and the glucosinolates. Both groups of compounds are widely distributed in the

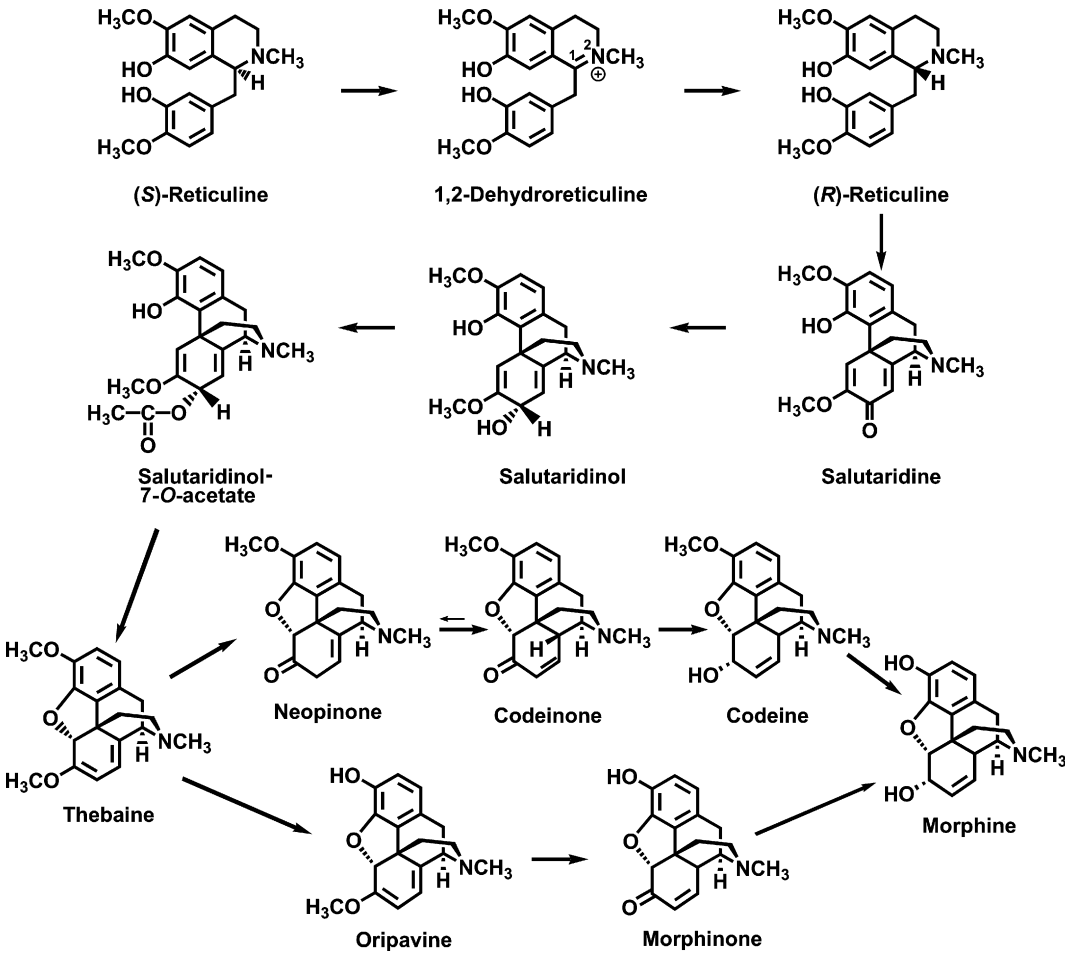

Fig. 11. The enzyme catalyzed formation of morphine from (*S*)-reticuline.

plant kingdom. The cyanogenic glucosides occur in ca. 1 % of all 300,000 plant species and in many plant families. If plant parts containing these glucosides are eaten or even damaged the cyanogenic glucoside is hydrolyzed by glucosidases and the labile aglycone will release poisonous HCN and aldehydes. This clearly points to a very effective defense compound keeping away grazing mammals and other predators from plants. In the mid 19th century, the city center of London was heavily planted with sycamore (*Platanus* sp.) trees. A panic among citizens arose when it was reported in the press that one single sycamore leaf releases an amount of HCN that can kill a sparrow. The trees were blamed for the observed sudden death of citizens, which however most likely was due to carbon monoxide from chimneys and stoves. One of the prominent members of the cyanogenic glucoside family is dhurrin, a major product in *Sorghum bicolor*. The pathway from l-tyrosine to dhurrin comprises only three enzymes that form a metabolome, a multienzyme complex that facilitates channeling of the intermediates in dhurrin synthesis. The enzymes are two multifunctional cytochrome P450 enzymes and one glucosyl transferase (Fig. 12a). In a formidable piece of work, the Lindberg Møller group (Tattersall et al., 2001) succeeded in transferring the dhurrin

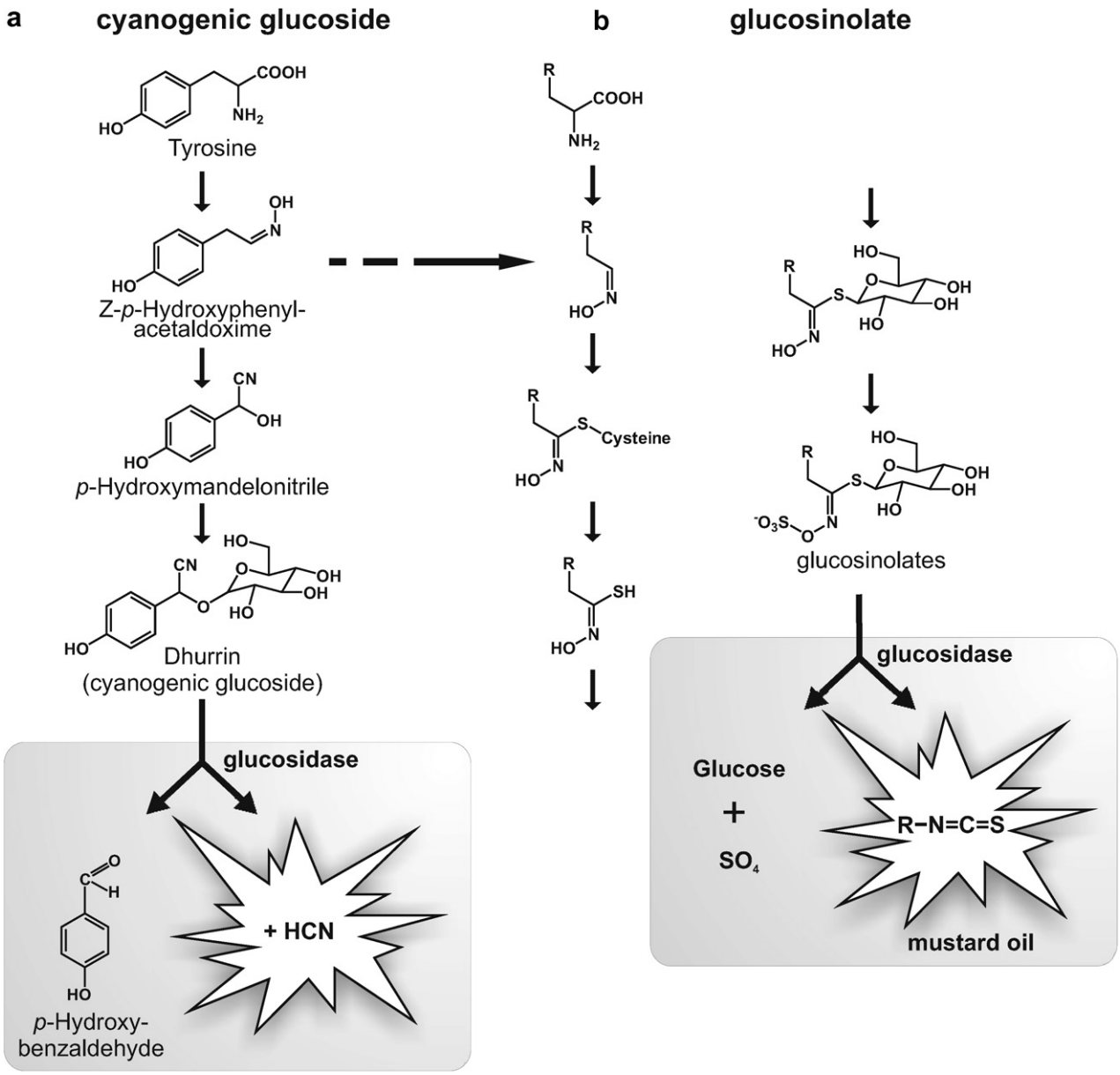

Fig. 12. (a) The formation of cyanogenic glucosides (e.g. dhurrin, (a)) and of glucosinolates (b) both originating from amino acids. Cyanogenic glucosides are biosynthesized in a metabolome containing only three enzymes and a cytochrome P450 reductase. The formation of glucosinolates from the aldoxime intermediate takes place by a multistep enzymatic sequence. If a plant is wounded by a predator, gluosidases act upon cyanogenic glucosides to produce poisonous hydrogen cyanide (a) or upon glucosinolates to release toxic mustard oil (b). (Modified from Kristensen et al., 2005).

metabolome from *Sorghum* to *Arabidopsis*. Dhurrin does not occur naturally in *Arabidopsis*. The genetically engineered *Arabidopsis* synthesizes up to 4% dhurrin per dry matter. The presence of dhurrin in the transgenic *A. thaliana* plants confers resistance to the flea beetle, which is a natural pest of other members of the crucifer group, demonstrating the role of cyanogenic glucosides in plant defense. With a similar cloning strategy, the same group (Bak et al., 1999,2006) succeeded in elucidating the glucosinolate pathway at the enzyme level, and isolating and functionally expressing the biosynthetic cDNAs in *Arabidopsis* plants. The transformed plants accumulated specific glucosinolates that did not normally occur in the wild type plant. Glucosinolates are also assumed to play a role in plant defense. Hydrolysis of glucosinolates by glucosidases releases toxic mustard oils (Fig. 12b).

4. Nonprotein amino acids

Next to the enormously large class of nitrogen-containing compounds, the alkaloids, with more than 10,000 identified and chemically characterized compounds and the glucosinolates and cyanogenic glucosides, there exists a small class of about 250 nonprotein amino acids which have been thoroughly investigated by Fowden et al. (1979). A small group of these amino acids is shown in Fig. 13. There is little information on the biosynthesis of these compounds, but some of them may arise by transamination to ketofunctions of appropriate precursors and further biochemical transformation. All of these nonprotein amino acids are toxic or exhibit antimetabolic properties and seem to be restricted to groups of closely related species, thereby serving as good chemotaxonomic markers.

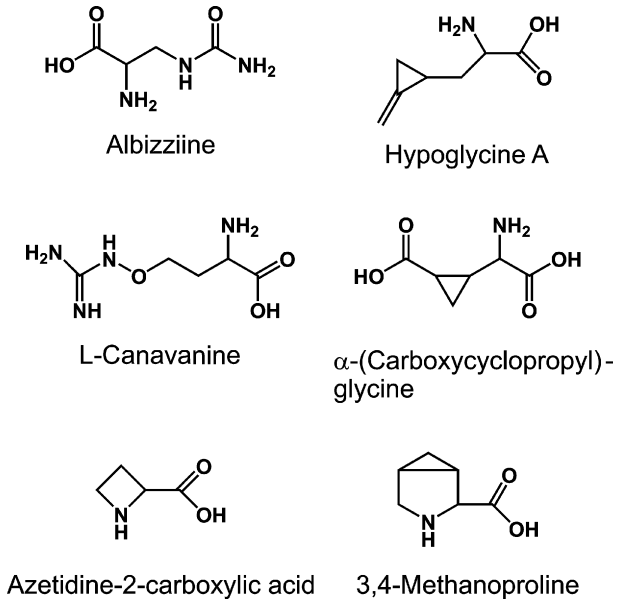

Fig. 13. Select structures of nonprotein amino acids.

Exceptions are, for instance, azetidine-2-carboxylic acid which is not confined to the *Liliaceae,* but occurs in legumes, in a red algae, in sugar beet and in beech!

The functions of these antimetabolites are not clear. It is likely that they suppress the growth or activity of a second species be it animal, microbial or plant. The mechanism whereby the producer species and other plants exhibiting resistance avoid toxicity is unclear. This poses intriguing problems on transport specificities, detoxification, compartmentation and enzyme structure (Fowden et al., 1979). To discover these mechanisms of resistance will be an attractive target for phytochemistry. Early on, one may have suspected that these antimetabolites could play a role in medicine. The fact that Sir Leslie (Fowden) isolated from 100,000 tons of sugar beet several kg quantities of azetidine-2-carboxylic acid by passing the extract through a gigantic cation-exchange column indicates that, while isolation is possible, this is an example in which synthesis might present a more realistic approach to obtaining large amounts of compound.

5. Conclusions

The study and use of alkaloids over the past 200 years is a success story. No other group of plant-derived compounds could compete with the alkaloids and their semisynthetic derivatives such as morphine, quinine, taxol, taxotere and many others that have not yet reached the status of blockbusters. Alkaloids are indispensable for modern human mankind and will remain so in the future. This success was reached by the interaction of many disciplines, and also by scientists who had a wide scope, took risks and had management skills to bring an alkaloidal product to commercial fruition. Phytochemistry, with its interdisciplinary nature, brought about this success. Nature is rich with the most outstanding bioactive molecules and biocatalysts; it is the responsibility of the generations to come to harvest these molecules. The projected shortage of traditional resources will have to introduce a change in our thinking towards renewable processes. As phytochemists, we should consider transgenic plants that will synthesize custom designed molecules by recombinant technologies using engineered enzymes with new specificities and properties (e.g. stability, K_m, turnover numbers, etc.) and appropriate downstream processes. Phytochemistry will surely have a bright future.

Acknowledgements

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