WORKSHOP ON BIOTECHNOLOGY OF STEROID COMPOUNDS AS CONTRACEPTIVES AND DRUGS

Summary Report

Jakarta, Indonesia
December 15–17, 1986

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PREFACE

In 1986, the National Research Council of Indonesia (DRN) invited the Board on Science and Technology for International Development (BOSTID) to join it in sponsoring a workshop on the production of steroid compounds for contraceptives and drugs in Indonesia. This workshop was held in Jakarta, Indonesia, December 15-17, 1986.

The primary objectives of the workshop were to exchange information on and experience in implementing biotechnological techniques to produce steroid contraceptives and drugs, to discuss the prospects for the biotechnology needed to produce these substances from natural resources, and to set priorities for an action program in this area to enhance the national capability.

The rapidly expanding family planning program in Indonesia requires an increasing supply of contraceptives, including oral ones. The distribution of oral contraceptives has increased from approximately 1-2 million cycles in 1970 to over 65 million cycles in 1985. Given the current anticipated population growth and the over 25 million women in the childbearing age group, it is estimated that over 150 million cycles will be needed by the year 2000.

In the past the supply of oral contraceptives was, to an important extent, met by foreign assistance, but much of this assistance is now being terminated. Considering the importance of steroid contraceptives in the national family planning program as well as the high commercial value of steroid drugs, the government of Indonesia decided to strive rapidly for self-sufficiency in supplying these drugs.

Particular attention was given in the workshop to the utilization of indigenous natural resources. Workshop participants recognized, however, that total as well as partial synthesis must be adopted to achieve the objective of producing the necessary steroid compounds for contraceptives and drugs.

These activities were one activity in a larger program of cooperation between BOSTID and the Indonesian government. Begun in 1968, this program has featured a series of workshops on food policy, industrial and technological research, natural resources, rural productivity, manpower planning, marine algae biotechnology, biotechnology in agricultural development, and development of a science and technology information system. BOSTID's participation has been supported in the context of a science and technology loan from the U.S. Agency for International Development (USAID) to the government of Indonesia. The current two-year program with BOSTID calls for a number

of activities (panel discussions, workshops, follow-up activities, or small advisory groups) to be organized each year.

ORGANIZATION OF THE WORKSHOP

This workshop was organized by a steering committee under the sponsorship of the Indonesian National Research Council.

Dr. Didin S. Sastrapradja, Assistant (II) Minister for Research and Technology and Chairman of the National Committee on the Development of Biotechnology, opened the workshop on behalf of Dr. B. J. Habibie, Minister of State for Research and Technology and Chairman of the DRN (Appendix A). In his keynote address, Dr. Sastrapradja described the long relationship between Indonesia and the U.S. National Research Council and its cooperative programs in the field of biotechnology and related subjects. Dr. Margaret Bonner, Acting Director of the USAID mission in Indonesia, commented on the importance of these kinds of exchanges in establishing strong scientific ties between Indonesia and the United States. In addition, she noted that this workshop will affect an area of long-standing USAID interest in Indonesia--family planning (Appendix B).

Part I of this report is composed of papers prepared for the workshop. Following presentation of these papers, participants broke into three working groups that addressed:

1. Sitosterol sources from agricultural by-products and natural resources

2. Production of steroid compounds by plant cell and tissue culture

3. Production of steroid compounds by fermentation and chemical synthesis.

A summary of the conclusions and recommendations of these working groups is presented in Part II of this report. Part III contains papers prepared by various U.S. and Indonesian participants that address the topics covered by the working groups.

On the final day of the workshop the conclusions and recommendations of the three working groups were presented by the chairperson of each group. Dr. Monroe E. Wall, chairman of the U.S. National Research Council (NRC) panel, spoke on behalf of his U.S. colleagues about the various steps needed for Indonesia to further develop its capability in steroid production. Closing remarks by Dr. Sastrapradja, the workshop agenda, and a list of the participants are included as Appendixes C, D, and E to this report, respectively.

This workshop report was prepared by Rose Bannigan of the BOSTID staff using papers written by the Indonesian and NRC workshop participants. The papers have been edited to eliminate duplication, but they accurately reflect the discussions. The final draft was reviewed and approved by the members of the NRC panel and the Indonesian organizing committee. Sabra Bissette Ledent, BOSTID consultant, edited the report. The participants would like to thank the members of the workshop secretariat for the excellent organization of the workshop.

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PART I

Presentations

DEVELOPMENT OF STEROID COMPOUNDS AS A RAW MATERIAL FOR DRUGS

Utarto
Director, PT Kimia Farma

USE OF STEROID DRUGS IN INDONESIA

Oral contraceptives play an important role in the family planning program run by the National Family Planning Coordinating Board (BKKBN) in Indonesia, although a variety of other contraceptives are also available. The BKKBN family planning program distributes contraceptive devices at no charge, thereby dominating the distribution of contraceptives nationally. Those obtaining contraceptives from the private sector must, of course, pay for the devices--including services and controls--themselves. This balance will change gradually in the future, however, because government spending will be limited. It is expected that the distribution of free contraceptive devices will decrease, while the distribution of partially subsidized and fully commercial drugs will increase.

Unfortunately, the ability of Indonesians to purchase contraceptives is still limited, making the overall situation of the family planning program a gloomy one. Obtaining help from abroad through grants or soft loans may still be possible, but heavier government expenditures must be avoided. All these factors will undoubtedly lead to the development of family planning activities in the private sector, especially among privileged communities such as those living in the cities, and to the urgent need to produce the raw materials for oral contraceptives, thereby saving foreign exchange as well as creating new sources of income and new jobs.

In the past, oral contraceptives for the BKKBN family planning program were largely acquired through grants from abroad. As a consequence, the kinds of pills obtained depended on such factors as which countries were willing to give a grant or loan for the program, what types of pills were available in those countries, and whether the pills provided and those who used them were compatible.

It was largely because of these factors that the first oral contraceptives distributed in Indonesia by the family planning program were Noriday 50 (considered a high-dosage pill) and Ovostat (distributed on a limited basis). These factors also explain why Noriday has been manufactured locally by Kimia Farma since the early 1980s.

More effective low-dosage pills with fewer side effects are now being distributed, but the family planning program found that the shift from high- to low-dosage pills was not as easy as expected because of budgeting problems. The most difficult problem, however, was that consumers were hesitant about changing their usual contraceptive for a new one. The family planning program is presently using two types of low-dosage pills, especially among new participants. They are levonorgestrel 150 micro/Eth estradial 30 micro and desogestrel 150 micro/Eth estradiol 30 micro. The latter drug, manufactured by Organan, is known as Marvelon. It is being used by the family planning program through a grant from the Dutch government.

In 1985-1986, the BKKBN family planning program distributed the following contraceptive devices: IUDs, 34 percent; oral contraceptives, 43.9 percent; injection drugs, 14.2 percent; condoms, 3.5 percent; and others, 4.4 percent. The injection drugs used by the family planning program consist of steroids. Thus, 58.1 percent of the contraceptives distributed are based on steroids, excluding Norplant (susuk KB).

Injection drugs are surprisingly well accepted by participants in the family planning program, and use of these drugs is increasing from year to year. The dominant drug distributed is Depo-Provera (Upjohn)--about 8 million vials per year. Its active ingredient is medroxyprogesterone acetate. Norethisterone enanthate, another injection drug, is also being distributed, but in smaller quantities. The weakness of this drug is that it is a kind of oily solution with a shorter interval of injection (one month).

The family planning program is also using Norplant, a recent invention. A plastic needle containing levonorgestrel is planted subdermally in the hand for a time-release of five years. About 20,000 units of Norplant are used each year, making Indonesia the largest consumer of this kind of device.

DEMAND FOR RAW MATERIALS FOR STEROID PRODUCTION

Projections of the future needs for oral and other contraceptives based on steroids (taking into account the future emphasis on low-dosage pills) are shown below. The projected first-stage demand for raw materials for steroid production follows:

Contraceptive	Projected needs
Noriday 50	20 million cycles/year
Norminest	10 million cycles/year
Microgynon	40 million cycles/year
Marvelon	40 million cycles/year
Depo-Provera MPA	10 million cycles/year
Norplant	20,000 units

Raw material	Projected needs (kg/yr)
Norethindrone	400-600
Mestranol/Ethynl estradiol	80-100
Levonorgestrel	150
Desogestrel	1,500

The demand for these materials will probably increase 5-10 percent each year.

VARIOUS RESEARCH ACTIVITIES

The first seminar on steroid compounds for contraceptives was organized in June 1981, under the sponsorship of the BKKBN. The participants included speakers representing various foreign companies such as Schering, Organon, Syntex, and Gideon Richter, as well as a number of experts from the National Institutes of Health (NIH).

Several local participants reported on their research aimed at finding suitable precursors, similar to those of diosgenin and solasodine.

A team from Bogor Agricultural University (IPB), who conducted a survey on the possibility of obtaining Dioscorea with a high diosgenin content, also reported on their progress at the seminar. Subsequently, they reported a negative result for their survey.

Finally, another team of researchers, headed by Prof. Oei Ban Liang, reported on experiments on the culture of Solanum khasianum. An analytic method was used to measure the solasodine content. In the meantime, the results of experiments on Solanum marginatum and Costus spp. have also been reported.

In the years following the first seminar, researchers have focused on finding sources of sterols in the agricultural wastes produced in Indonesia. For example, Lubis and associates have successfully identified and isolated sitosterol from pressmud (sugarcane waste) with a content of 0.32-0.83 percent. Further study must be undertaken to determine the economic value of this resource.

Another possible source of sterol is the oil obtained from soybean processing. This possibility must be studied in terms of its economic value, however.

The application of biotechnological processes to the transformation of sitosterol to ADD (androsta-1,4-diene-3,17-dione) is a breakthrough that dramatically changes the conventional synthesis process and alters the pattern of the steroid industry. Because production of ADD from diosgenin via the conventional method involves many stages, that method has been abandoned. Nevertheless, steroid precursors such as diosgenin and solasodine are still useful for the production of corticosteroid using a synthesis process.

INDUSTRIAL EFFORT

Since the 1981 seminar, many foreign companies have been contacted to explore cooperative efforts and the transfer of technology in the area of contraceptives. Some companies have shown great interest in the matter, while others have been reluctant or even refused to participate to maintain their own self-interests.

Kimia Farma has adopted a strategy to gain cooperation in producing a steroid contraceptive within the stage of forward integration activities. This will be followed by an effort to handle the backward integration activities step by step, while also gaining the technology and trained manpower needed to manufacture sitosterol, diosgenin, and solasodine, for example.

The first calculations have shown that the "transfer price" of products manufactured locally (that is, in cooperation with foreign companies using their technological capability and expertise) is 135-400 percent higher than that of products that can be bought on the world market. Such a high price results from economies of scale and the transfer price of the starting materials or other intermediates. On the one hand, the transfer price must be borne by the project in favor of Indonesia receiving this technology. On the other hand, too high a price cannot be tolerated. This problem must be faced in participating in a joint venture for a sophisticated technology. It is therefore time to look for ways to develop our own technology using channels of scientific cooperation and an exchange of experience as well as knowledge.

This process will take a long time just to establish, and from a business perspective this may mean losing good opportunities. It is hoped that this workshop will provide the necessary input as well as momentum to motivate the development of steroid compounds in Indonesia.

EXTRACTION AND BIOTRANSFORMATION STUDIES OF STEROIDS AND MORPHINAN ALKALOIDS FROM INDONESIAN BIOLOGICAL RESOURCES

Ischak Lubis and Susono Saono
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INTRODUCTION

Many raw materials and precursors used in the manufacture of valuable drugs are derived from plants. The importance of plant products in the pharmaceutical industry has become even greater since the successful manufacture in the early 1960s of the steroid drug diosgenin, a plant steroid then obtained from Dioscorea and later from other plants as well.

The incorporation of biotransformation by microorganisms or by tissue culture has significantly increased the economic prospects of exploiting various plant products to produce useful drugs. An important example of this trend is the application of the microbial transformation of phytosterols into androstene-3, 17-dione (AD) and androsta-1, 4-diene-3, 17-dione (ADD), important intermediates in the manufacture of contraceptive drugs. Because microbial transformation is much more efficient than the chemical synthesis originally applied in the industrial manufacture of contraceptive drugs from diosgenin, and the raw materials needed are relatively cheap, the microbial transformation process is preferred. Much attention has thus been given to searching for possible sources of suitable phytosterols (that is, stigmasterol and sitosterol) and potential strains of microorganisms for the transformation.

In a process first introduced by Schering AG, soybean waste after oil extraction has served as an inexpensive source of stigmasterol and sitosterol, but other sources may be available. Thus, oil-producing plants as well as a number of agricultural wastes abundantly available in Indonesia must be examined as possible sources of sterols. It is also necessary to select potential microbial strains capable of transforming sterols into steroid drug intermediates, particularly AD and ADD.

Other important drugs of considerable economic and therapeutic value are the morphinan alkaloids (codeine, morphine, papaverine), which are produced commercially from Papaver somniferum. Abuses of narcotic alkaloids for nonmedical purposes have, however, made it necessary to put strict controls on their production. It would thus be ideal to devise a method for producing the alkaloids morphine and codeine from nonnarcotic starting materials.

As total synthesis is obviously impractical and expensive, the search for plant products usable as precursors for the synthesis of codeine and morphine has been attempted. In this connection, studies on the occurrence of reticuline, an isoquinoline alkaloid frequently found in Annonaceae, and on its chemical conversion, have been reported by various investigators. Using nonnarcotic salutaridin as a key intermediate, researchers have now established the synthetic pathway into thebaine. Recently, our institute isolated salutaridin from an Indonesian plant, leading us to explore the possibility of using this alkaloid for the production of codeine and morphine. The main problem is transforming the salutaridin into thebaine, from which codeine and morphine can then be produced by known chemical processes. If this could be achieved, there would be no need to cultivate Papaver somniferum, and the abuses of the opium and its narcotic alkaloid would be drastically minimized.

CURRENT RESEARCH PROGRAM

Scientific and Economic Justification

The synthetic pathway of various steroid drugs, including oral contraceptives, has been established. Commercial application of this synthesis has been improved from time to time, especially after the utilization of microbial transformation to produce the major intermediates.

AD and ADD are the primary intermediates for the production of contraceptive drugs. If these intermediates can be produced commercially, the subsequent processes to produce the desired steroids will be much easier. Thus, the main problem is the production of ADD from a suitable sterol as precursor. Because β-sitosterol is one of the most widely distributed phytosterols, the availability of this sterol from local plants should be determined, as well as the occurrence of sitosterol in certain agricultural wastes. Primary attention has been given to the sugarcane waste pressmud, as sugarcane production is an important agricultural industry in Indonesia. The economic value of sugarcane will be enhanced even further if sitosterol is obtained as an additional by-product.

The availability of sitosterol from sugarcane waste in this country, coupled with the application of microbial transformation, would be an effective approach to starting industrial production of steroid drugs, especially oral contraceptives. Self-sufficiency in the production of contraceptive drugs will in turn save foreign exchange now used to import contraceptive compounds.

Experiments have been carried out to isolate sitosterol from pressmud obtained from sugarcane factories in East Java. Extraction methods have been compared to find the most efficient method. A greater yield of purified sitosterol was obtained from raw material previously fermented (by kipsen in water) to destroy waxes and proteinous compounds. Using this method (see Annex A to this paper),

620 mg of sitosterol was obtained from 75 g of dried fermented pressmud (about an 0.83 percent yield). Further studies are still needed, however, to increase the yield. Possible steps include improving fermentation of the pressmud and applying a locally available extraction solvent such as acetone which can also be produced from molasses. It is hoped that the production of sitosterol from sugarcane waste can be carried out inexpensively.

A preliminary study has also been conducted on screening microbial strains for the transformation of sitosterol into AD and ADD. As many as 3,000 strains have been tested, but further experimentation is necessary to find the potentially effective microorganisms. This research will focus on strains obtained from culture collections or natural habitats, including Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Mycobacterium, Nocardia, Serratia, and Streptomyces. Collaboration with other institutes is obviously needed to achieve the desired results in the shortest possible time.

In addition to the selection of effective organisms, studies on fermentation conditions will be conducted for most potential strains to obtain the optimal yield of bioconversion. A standard chemical analysis using high-pressure liquid chromotography (HPLC) will be employed to monitor the process.

The possibility of synthesizing morphinan alkaloids from the nonnarcotic alkaloid salutaridin seems to be within reach of present chemical or biochemical knowledge. Synthesis of salutaridin to thebaine is expected to consist of only a few steps, thus facilitating industrial production. Isolation of salutaridin from a native plant (using leaves) involved column chromarographic separation and crystallization from benzene. A yield of 0.28 percent was obtained from the leaves, and as this native tree produces abundant leaves, the alkaloid content would be sufficient for possible commercial exploitation. (A detailed report of the isolation and characterization of the alkaloid is given in Annex B to this paper.)

PLANNED SHORT-TERM RESEARCH PROGRAM

Sitosterol for Steroids

As of fiscal year 1987-1988, plans include conducting studies on the extraction of sitosterol from sugarcane waste, its biotransformation by microorganisms, and fermentation conditions to achieve optimal conversion of sitosterol into AD and ADD. Studies will also be carried out on the sitosterol contents of other agricultural wastes, especially palm oil residue, and of other oil-producing plants such as Euphorbia which can be grown on poor soils with minimal maintenance. The program on contraceptive and steroid drugs is expected to yield sufficient data for initiation of a semipilot- or pilot-scale experiment within two to three years.

Plant Cell and Tissue Culture for Crop Improvement and Secondary Metabolites Production

Studies on how to improve both the productivity of fruit crops and products of medicinal value will be conducted by employing plant cell and tissue culture techniques. Low productivity and pest and disease sensitivity are problems afflicting Indonesian agricultural commodities, especially fruit crops. The problems encountered in utilizing plant resources are the lengthy period required for crop improvement using conventional techniques and the low production of vauable compounds. It is hoped that the improved quality of the fruit crops selected for study in the coming fiscal year will increase productivity substantially.

To achieve the research goals described above, all interested research workers from the various research institutes, universities, and related industries must join together as an integrated team to undertake these studies. It is expected that this workshop will provide the opportunity to work out the basic mechanism of cooperation among the interested institutions.

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ANNEX A

Sitosterol from Sugarcane Waste

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INTRODUCTION

Sitosterol is one of the most widely distributed phytosterols of the higher plants. Found mostly in small quantities, sitosterol and other major phytosterols (stigmasterol and campesterol) may occur in certain legume and latex-producing plants, such as Euphorbia and Calotropis, in relatively greater concentrations.

The occurrence of sitosterol in higher plants recently attracted attention when Schering AG introduced the industrial microbial conversion of sitosterol into steroid intermediates AD (androstene-3, 17-dione) and ADD (androsta-1, 4-diene-3, 17-dione) for the manufacture of contraceptive drugs. Soybean waste after oil extraction has served as a commercial source of sitosterol for this process. Because sitosterol obtained from soybean waste is very inexpensive, this phytosterol has become a major precursor for steroid production. In searching for other possible commercial sources of sitosterol, researchers are focusing on certain agricultural wastes, especially the waste from sugarcane known as pressmud (blotong in Indonesian).

Annual biomass production in Indonesia amounted to more than 19 million tons in 1984. Thus, it is obvious that the sugarcane industry is producing abundant unused pressmud. If sugarcane waste constitutes 5-10 percent of the total biomass, the unused residue would amount to 1-2 million tons annually. It would then be useful to investigate the phytosterol content of pressmud.

This paper reports on the extraction yield of sitosterol from pressmud using the medified method developed by S. K. Srivastava et al. (1985).

MATERIALS AND METHODS

Pressmud with a 70-80 percent water content was obtained from a sugarcane factory in East Java. Extraction was conducted by rinsing the dried pressmud (with and without previous fermentation treatment) in water in a scaled bottle for five weeks. The treated sample was then dried at 80°C and ground into a fine power of which 100 g was refluxed with a mixture of benzene, petroleum benzene 40-60, and

ethanolic 2N KOH (10:5:1) for 12 hours. The extract was then distilled under reduced pressure and the concentrated residue refluxed for 30 minutes in acetonitril (2 x 50 ml), which was decanted while hot. After standing overnight, the precipitated white product was filtered and purified using hot isopropanol.

A yield of 0.036 percent (100 g yielded 36 mg of sitosterol) was achieved using dried unfermented pressmud. Fermentation of the pressmud, however, resulted in an 0.83 percent yield (75 g yielded 620 mg of sitosterol).

CONCLUSION

It can be concluded from these results that the sugarcane waste pressmud is a potential source of sitosterol. Moreover, it was proven that fermentation of pressmud increases the yield of extraction. Further improvement of the extraction techniques used as well as utilization of an inexpensive solvent (such as acetone obtained from molasses), are now required.

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ANNEX B

Manufacture of Codeine and Morphine from Salutaridin, a New Alkaloid from an Indonesian Plant

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INTRODUCTION

Morphine and codeine—the major alkaloids of *Papaver somniferum*—are quite valuable medically. The production and distribution of opium and its morphinan alkaloids are under strict international controls, however, because abuse of these alkaloids for nonmedical purposes can poss a serious health threat. This problem has stimulated attempts to produce morphine by means of total and semisynthetic processes that use nonnarcotic compounds as a precursor.

Reticuline, an isoquindine alkaloid, is widely distributed among members of the *Annonaceae* family of tropical plants, which are commonly found in Indonesia. Biosynthetic studies of the *Papaver somniferum* alkaloids have revealed that reticuline acts as a natural precursor (Battersby et al., 1964; Barton et al., 1965; Waddel and Rapoport, 1985). It was also evident from radioactive labeling that reticuline was converted into salutaridin by a selective oxidative coupling (paraortho of the A and C rings). Salutaridin was then converted into thebaine from which codeine and morphine can be produced.

The revelation that reticuline can be used for the synthesis of morphine has stimulated studies on in vitro oxidative coupling of reticuline using various oxidative agents. It has been found, however, that in vitro conversion of reticuline into salutaridin is not very efficient, giving only a 0.03 percent yield (Hewgill and Pass, 1985). It would be more practical to use salutaridin, not reticuline, as the potential precursor for the synthesis of codeine and morphine.

The occurrence of salutaridin was first reported in 1985, when it was found in *Croton salutaris* (*Euphorbia*) growing in Brazilia at 2,000 m above sea level. Studies by the Center for Research in Biotechnology on the alkaloids found in the *Annonaceae* family in Indonesia revealed that salutaridin is found in one species that is widely distributed in the forests in Sumatra and Kalimantan.

This paper briefly describes the isolation and identification of the alkaloid salutaridin. It is the first report of the occurrence of salutaridin in *Annonaceae*.

ISOLATION AND IDENTIFICATION OF SALUTARIDIN

In this examination of salutaridin, 300 g of dried powder of leaves were extracted, using 2 l of 95 percent alcohol containing 10 percent concentrated ammonia. Extraction was performed at 70°C for two hours, and the extract was filtered through filter paper (this was repeated three times). The alcoholic extract was then combined and concentrated to about 50 ml with a rotary evaporator. The residue was dissolved in 50 ml HCl IN, and the solution was then defatted with petroleum-ether, made alkaline (pH = 8.5) with ammonia, and shaken with chloroform (5 x 150 ml). At this point, the combined chloroform extract was concentrated into a viscous liquid, which was separated by column chromatography on aluminum, using benzene and benzene-ether mixtures with ratios of 9:1, 8:2, 7:3, 6:4, and 1:1 subsequently as eluents. Benzene-ether mixtures of 8:2 and 7:3 gave a single spot on thin-layer chromatography. These mixtures were then combined and concentrated, and a fine crystalline needle (melting point, 206-207°C) was obtained. Spectral analysis (IR, H NMR, ^{13}C NMR) and mass spectroscopy revealed that the alkaloid was salutaridin, $C_{19}H_{21}NO_4$.

A yield of 0.28 percent was achieved in this study—that is, 300 g of leaves yielded 850 mg of salutaridin.

CONCLUSION

The discovery of salutaridin in annonaceous plants in Indonesia may provide the opportunity to produce morphine and codeine without having to grow Papaver somniferum, the commercial source grown legally in certain countries. The role of salutaridin as a biosynthetic intermediate closer to thebaine than reticuline should be explored to determine the possibility of using salutaridin as an artificial precursor. If it is found that salutaridin is convertible to thebaine, then codeine, and subsequently morphine, can be obtained by known chemical synthesis. This would serve as a breakthrough for the pharmaceutical industry and it would minimize drastically the abuses of opium.

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PART II

Conclusions and Recommendations

SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS OF THE WORKING GROUPS

SITOSTEROL SOURCES FROM AGRICULTURAL BY-PRODUCTS AND NATURAL RESOURCES

Potential Major Sources of Phytosterols

The following major potential sources of mixed phytosterols are available in Indonesia. Not all of these sources will be practical, but all are available in sufficient quantities to be considered for use on an industrial scale.

Source	By-product
Sugarcane	Pressmud
Palm oil	Oil, kernel, presscake, fiber
Peanuts	Oil, presscake
Coconut	Oil, presscake, fiber
Rice	Presscake, rice bran oil
Cotton	Seed, presscake, oil
Ceiba spp.	Seed, presscake, oil
Ricinus spp.	Presscake, oil
Leucaena	Seeds
Soybean	Oil, presscake
Pinus (pine)	Tall oil

Of these sources, palm oil and sugarcane will receive the highest priority. In the case of the palm oil, major estates already exist and will be expanded. In addition, more are planned in the near future. It is expected that within five years Indonesia will be a major producer of palm oil.

In the case of sugarcane, 19 million tons of biomass are produced annually. The pressmud fraction, which is known to contain sterols and is currently discarded, represents 5-10 percent of the biomass produced, or 1-2 million tons annually.

Analysis

Analytical Methods

Nonsaponifiable Fractions. The major compounds of interest in the projected studies will be found in the nonsaponifiable fraction of the various plant materials or oils. The initial material will be obtained therefore after evaporation of a solvent (diethyl ether, petroleum ether, or methylene chloride) at a low temperature (35°C-45°C) under vacuum. The residual material will be treated with methanolic KOH (or NaOH) using a standard procedure, refluxing for a specific time. After cooling, the methanolic solution can be diluted with water and extracted with diethyl ether, petroleum ether, or methylene chloride. The extract can be dried by shaking with anhydrous sodium sulphate and then partially concentrated, diluted to a specific volume, and stored under refrigeration in the dark. Precaution must be taken to prevent leakage of vapors and a possible fire hazard. Glass, plastic, or rubber stoppers should be used.

The nonsaponifiable fraction is analyzed as follows. An aliquot of the nonsaponifiable fraction is concentrated to dryness under nitrogen using a suitable vial (plastic must be avoided), derivatized with a reagent, and the vial is tightly sealed. It is then warmed according to standard procedures for trimethyl silylation of sterols. An appropriate aliquot is analyzed using standard procedures and an internal standard--for example, cholesterol. From the weight of an aliquot of the original extract and that of an aliquot of the nonsaponifiable fraction, one can calculate the percentage of nonsaponifiable fraction in the plant or oil or the percentage of sterol in the nonsaponifiable fraction.

Alternatively, an aliquot of the nonsaponifiable fraction can be analyzed by high-pressure liquid chromatography (HPLC) using the same internal standard (cholesterol) described above for the gas chromatography (GC) procedures. Appropriate normal or reverse phase columns can be utilized.

Tocopherol can be quantitatively determined colorimetrically by HPLC or gas liquid chromatography (GLC) as TMS ether.

Carotene can be determined in an aliquot of the nonsaponifiable fraction (Wall method) by column chromatography and, after concentrating to a convenient volume, it can be determined colorimetrically at 450 nanometers.

Isolation and Purification of Phytosterols from Various Plant Sources. The objective of a study on the isolation and purification of phytosterols from various plant sources would be preparation of a purified phytosterol fraction containing at least 80 percent sterol, predominantly sitosterol. The scale should eventually be sufficiently large (that is, using at least 3.2 l of oil or 1 kg of seeds) so that some realistic projections of yields can be made.

The following procedures should be established for a crude sterol:

- Preparation of crude sterol fraction from the free sterols in oil. The conjugate sterols also extracted from plant materials will yield another fraction of crude sterol. In this case, procedures would simply be scaled up from the analytical methods described in the preceding sections.

- Large-scale column chromatography of the nonsaponifiable fraction. Suitable large absorption columns using either florisil or alumina or silica gel can be studied. Various chromatographic fractions should be collected, using solvent of increasing polarity. All fractions should be analyzed for sterols and other potentially useful components as described in the preceding sections on analytical methods. Although this method may not be industrially practical, the information obtained will be valuable.

- Short path distillation. Sterol and tocopherol fractions from the nonsaponifiable fraction can be readily separated from lipids and higher molecular weight. The concentrated steroid fraction obtained can then be crystallized.

- Soybean sludge method. Detailed procedures for the treatment of soybean oil have been published. The method includes a deodorization treatment followed by steam distillation. When it is carried out on a large scale, an invaluable sludge—sometimes called soybean "foots"—is produced and collected. The sludge is further treated using existing methods.

Effects of Location, Season, and Variety on Phytosterol Content

It is well known that both the yield and the composition of seeds and their corresponding oils are affected by many factors. These include geographic location (seashore, mountains, etc.), altitude, soil, fertility conditions, climatic conditions (rainfall, temperature), and season.

Seeds, oil, and presscake should be collected during the appropriate time periods. Where possible, data on climatological factors, soil, and other conditions should be collected as well.

Proposed Program

One of the key features of Indonesia's strong program of family planning is the decision to produce steroid contraceptives from Indonesian plant resources. Presently, a reasonably successful program

is under way for the production of solasadine from Solanum. Solasadine can be converted to ADD (androsta-1, 4-diene-3, 17-dione) by well-known chemical methods. In the United States and Western Europe, methods are available for converting sitosterols to ADD in one step on an industrial scale.

Indonesia has a large number of plant sources, particularly palm oil and sugarcane (pressmud), from which large amounts of sitosterols can be extracted. Detailed information on the sitosterol contents of these and other oils available in Indonesia and methods for large-scale isolation are needed. A decision can then be made on whether sufficient sitosterol from Indonesian sources exists for conversion to ADD using a fermentation procedure.

The Ministry of State for Research and Technology should act as coordinating agency for this program.

Plan of Action

The resources listed in first section above should be analyzed for sterol content according to the proposed established standard procedures.

The following institutions should participate in this program: Bandung Institute of Technology, University of Padjadjaran, Bogor Agricultural University, Airlangga University, Gadjah Mada University, Biotechnology Research Center (Indonesian Institute of Sciences), Agency for the Assessment and Application of Technology (BPP Teknologi), and Kimia Farma.

Each institution should be represented on a coordinating board which will be responsible for research planning and allocating funding and facilities (equipment) to the participating institutions.

At the initial stage of the program an inventory should be made of the ongoing research, availability of equipment, and research capability in terms of trained personnel.

Research activities should include clear targets within a five-year period. The first three years would be devoted to analysis and laboratory-scale isolation and the last two years to scaling up experiments on extraction and conversion.

A periodic evaluation of the overall progress should be conducted at the end of the fiscal year, at which time each institution should submit an annual report.

Sufficient funds should be allocated the first year to make available at least one gas chromatograph, one HPLC, and one short-path still for all participating institutions, as well as the general laboratory glassware and chemicals needed for the tasks described here.

It is expected that U.S. institutions actively engaged in steroid research and development such as the Research Triangle Institute (RTI) will provide short-term training for the Indonesian scientists participating in this project.

PRODUCTION OF STEROID COMPOUNDS BY PLANT CELL AND TISSUE CULTURE

R&D and Production

Assuming that Indonesian sources of solasodine and diosgenin from plants are competitive on the world market, it appears that the best plant sources are: (1) Solanum spp. (Solanum khasianum and Solanum marginatum), and (2) Costus or Dioscorea. Comparative agronomic and critical analytical data should be developed to select the Solanum species to be grown in Indonesia and its most suitable geographic location.

Plant cell and tissue culture systems can be used for the propagation and improvement of field-cultivated plants. Collection problems (thorny leaves) and plant disease may be associated with Solanum species. Plant tissue culture is useful for micropropagating a Solanum clone of a plant selected from the field that contains, for example, a desired morphology, solasodine content, or disease resistance. Plant cell and tissue culture systems can also be used directly in the laboratory to select improved Solanum plants with the desired morphology, solasodine content, disease resistance, and stress tolerance.

A Solanum tissue culture system subjected to substrate, nutrition, growth regulator, and stress studies may be used to understand solasodine production or important steroid biotransformation processes.

Plant cell and tissue culture systems will also be useful for developing a cost-effective method for in vitro production of steroids. Presently, the production of steroids using plant cell and tissue culture is not cost-effective.

Finally, improved coordination and communication are needed among participants in the research and development of a steroid production program.

Manpower Development

Critical steroid analysis is needed. Competent personnel for GC/HPLC are now available in Indonesia, but there is a critical shortage of instruments, dependable electrical power, and supplies which should be corrected as soon as possible.

The minimum nucleus of personnel needed for plant cell and tissue culture biotechnology is presently available in Indonesia. Additional personnel are needed for all aspects of work in plant cell and tissue culture biotechnology, including plant improvement, suspension culture and scale-up, biotransformation, and germ plasm conservation.

U.S.-Indonesia Cooperation

Cooperative programs could be established on crop improvement and conservation with the universities of Colorado, Florida, Tennessee, Minnesota, and Wisconsin, and the U.S. Department of Agriculture, and on suspension culture, scale-up, and biotransformation with Cornell University, University of Tennesee, State University of New York, and University of Minnesota.

Indonesian government and U.S. funds must be acquired to support laboratory training and to obtain supplies, etc. Personnel sent abroad should receive support to visit U.S. laboratories and to attend U.S. national meetings.

PRODUCTION OF STEROID COMPOUNDS BY FERMENTATION AND CHEMICAL SYNTHESIS

This group first identified several classes of steroid compounds such as contraceptives, corticosteroids, anabolic agents, diuretics, and androgens, that must be produced domestically. The group felt that pricrity should be given to contraceptives, corticosteroids, and androgens, but not necessarily in this order.

The group also discussed the various pathways in the production of the three classes of steroid compounds, and determined the manpower, equipment, and annual operational budget needed to carry out the program. An agreement was also reached on some aspects of where the programs should be implemented.

Recommendations

It is recommended that efforts be made to produce the following steroid compounds as quickly as possible:

- Contraceptives: ethynylestradiol, mestranol, norethindrone, norethindrone enanthate, levonorgestrel, medroxyprogesterone acetate, and desogestrel
- Corticosteroids: prednisone and prednisolone, cortisone and hydrocortisone, and dexamethazone
- Androgens: testosterone and testosterone-like compounds.

The production of these steroid compounds should be carried out according to the following pathways:

- Total synthesis for levonorgestrel (Figure 1) and desogestrel (Figure 2).
- Partial synthesis from sitosterol to norethindrone and the estrogens (Figure 3).
- Partial synthesis from steroid sapogenins to medroxyprogesterone acetate, corticosteroids, and androgens (Figure 4).

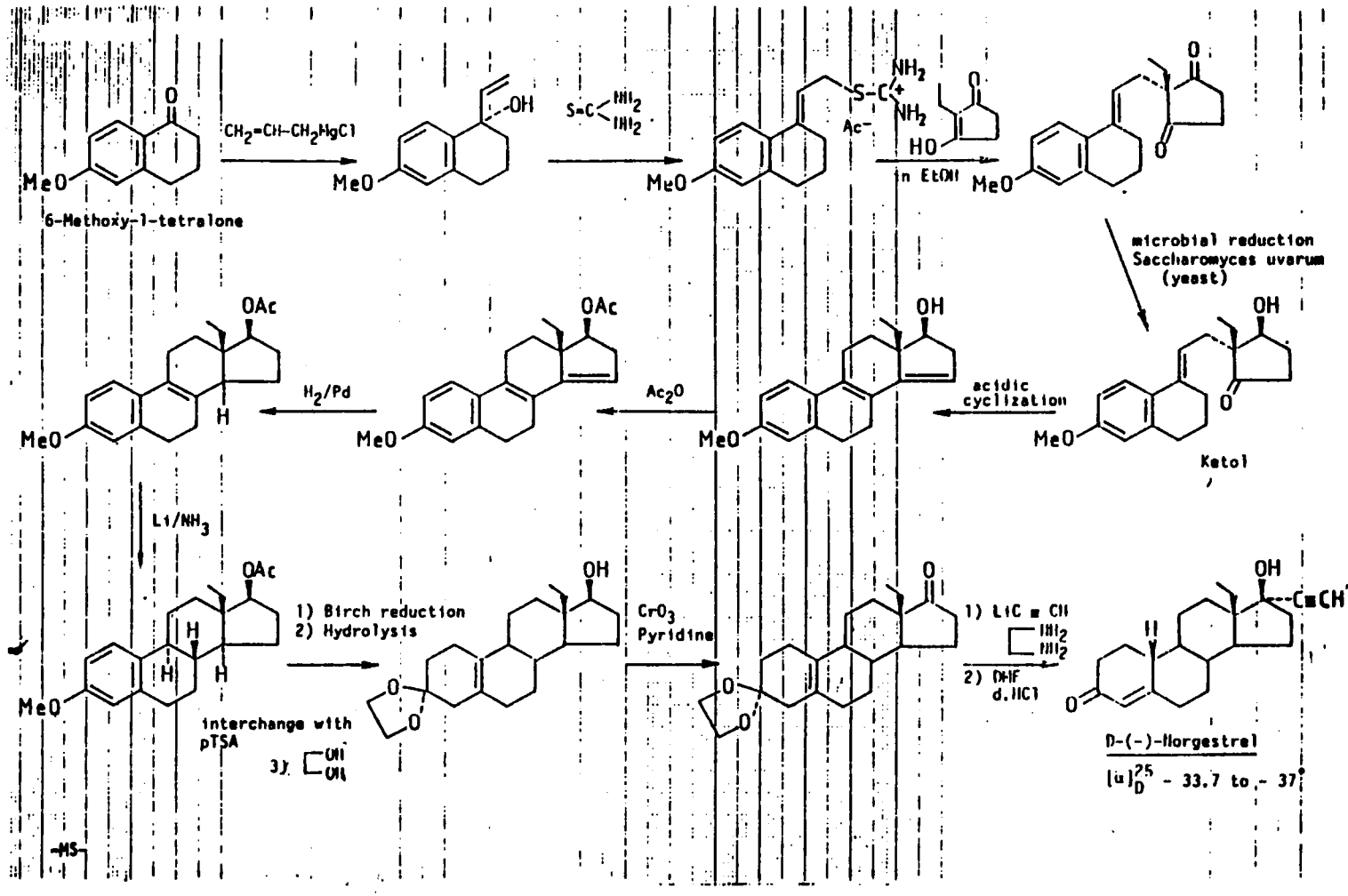


FIGURE 1 Total synthesis of D-(-)-norgestrel.

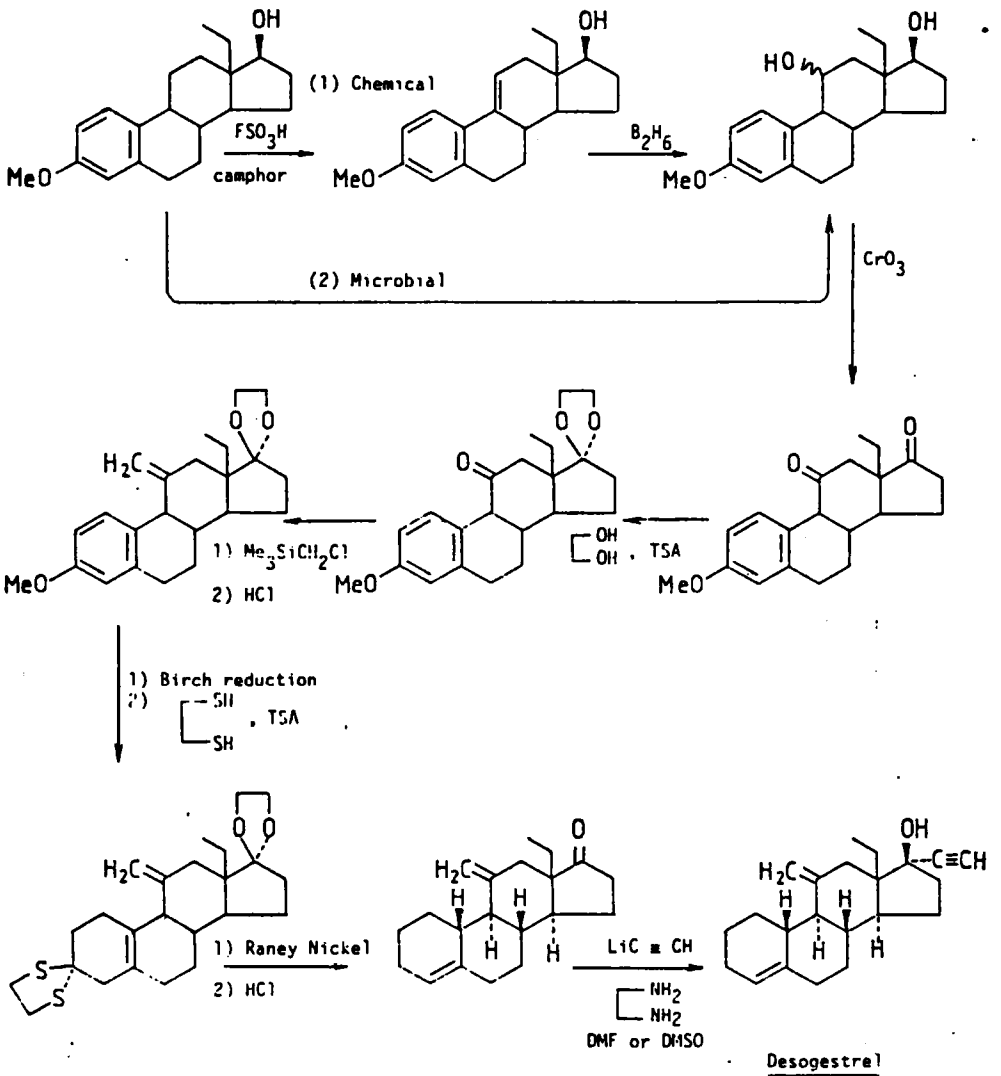


FIGURE 2 Synthesis of desogestrel from D-13β-ethyl-3-methoxygona-1,3,5 (10)-trien-17β-ol, a precursor of D-(-)norgestrel.

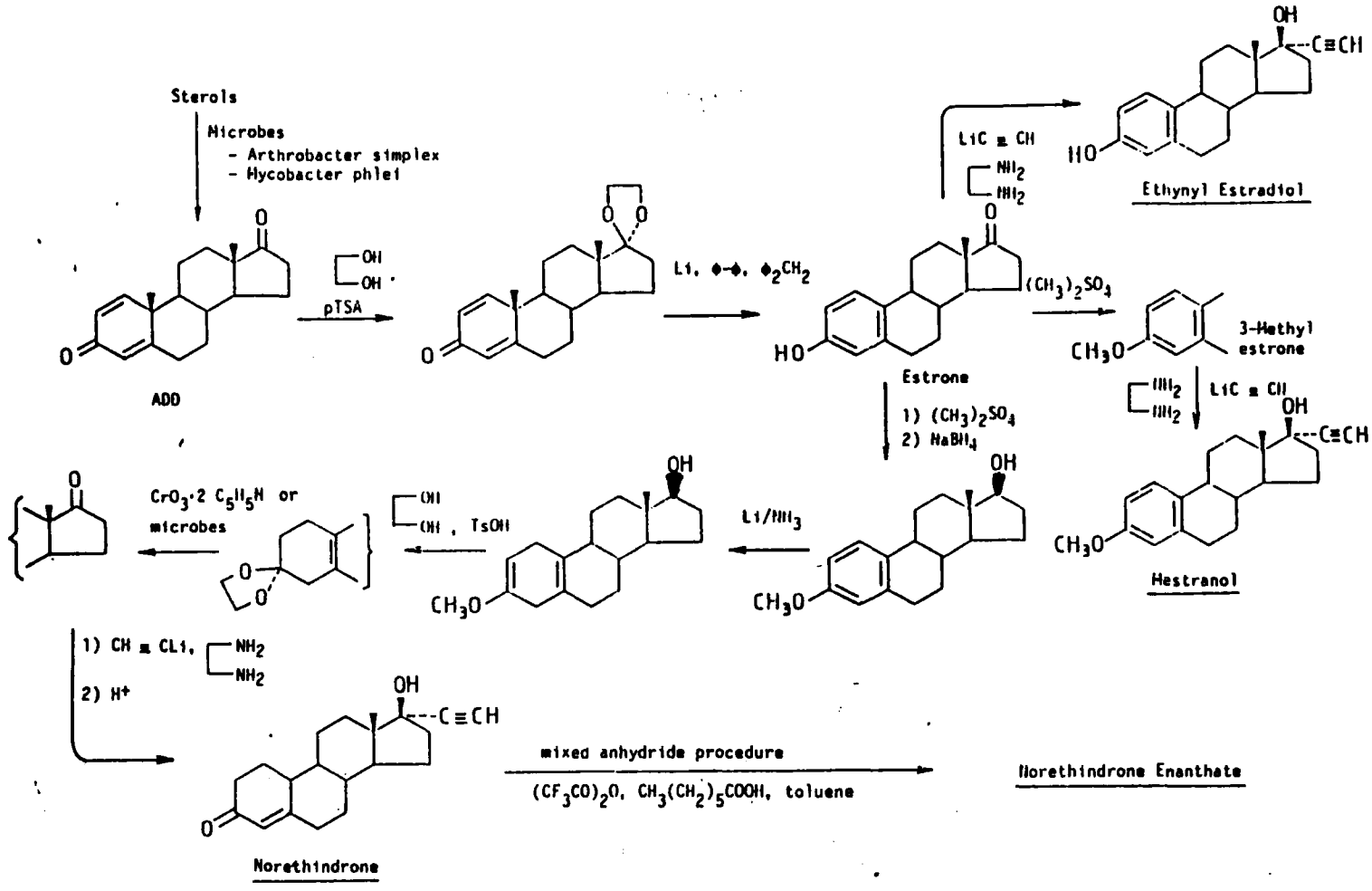


FIGURE 3 Partial synthesis of 19-nor steroids and the estrogens from sterols.

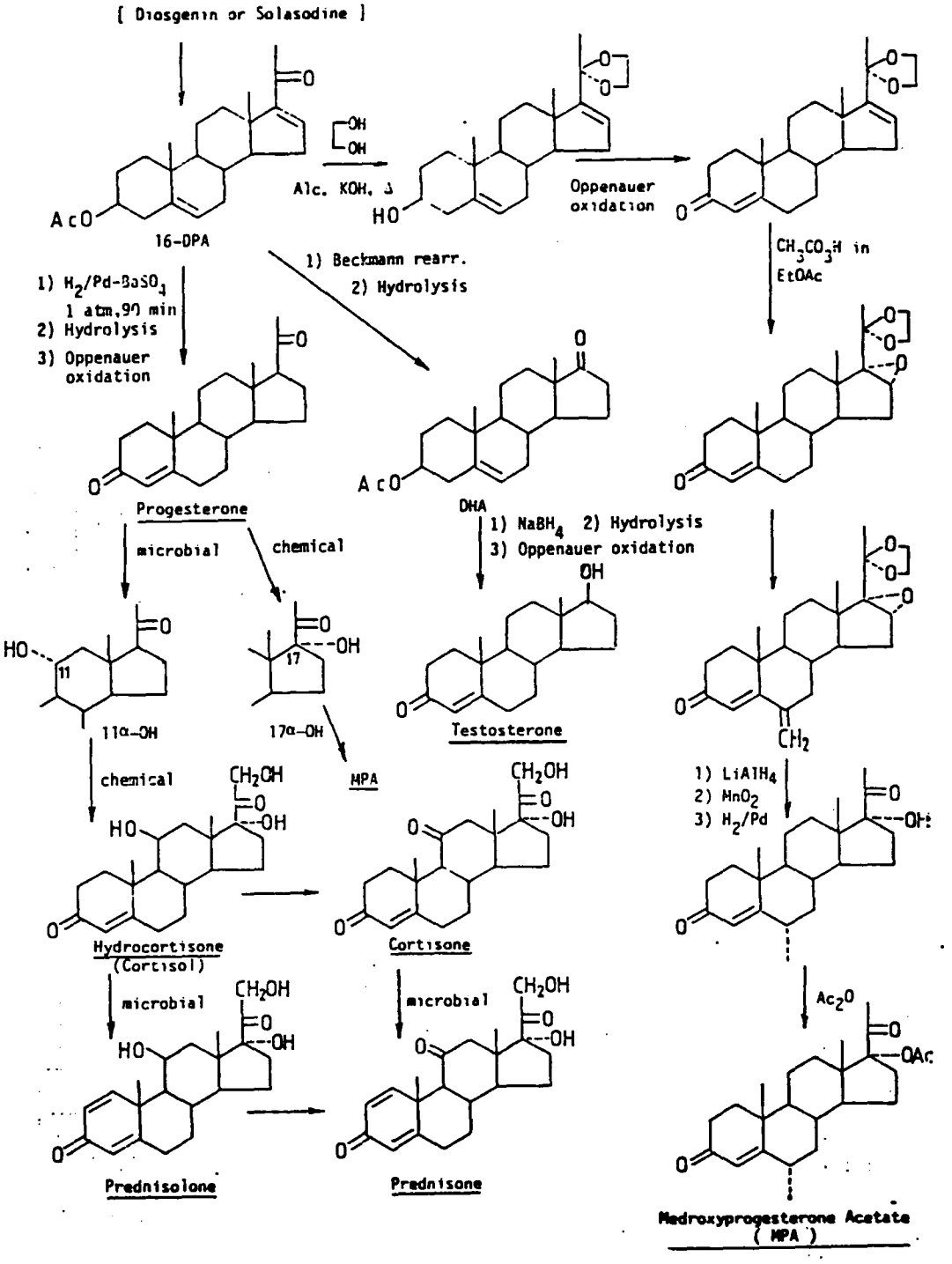


FIGURE 4 Partial synthesis of medroxyprogesterone acetate (MPA), testosterone, and corticosteroids from steroid sapogenins.

To initiate the program, sitosterol and optically active trienol should be imported; 16-DPA could be produced domestically. The microorganisms needed to carry out the microbial conversions could be obtained from established stock culture collections abroad.

The following personnel will be needed to carry out the three pathways: Ph.D. degree holders, 3; M.S. degree holders, 3; technicians, 9 (3 x 3); and administrative personnel, 2.

Table 1 lists the equipment needed for the program described here. An investment cost of US$1 million is anticipated. The annual budget for operation, including consumables and equipment maintenance, would be US$500,000.

TABLE 1 List of Equipment Needed

Equipment	Number
Rotary vacuum evaporator set (1 liter)	4
Rotary vacuum evaporator set (10-20 liters)	1
Freeze dryer	1
Sample dryer, vacuum and heater	1
Sample dryer, vacuum, dessicator	1
Automatic water distiller and demineralizer	1
Oven dryer	1
Oven dryer and incubator	1
Freezer and refrigerator for samples	1
Freezer dryer-lyphophilizer	1
Burner for glass blowing	1
Air compressor for glass blowing	1
Fraction collector	5
Semipreparative fraction collector	1
Fume cupboard set	1
Top loading balance	1
Analytical balance	1
Solvent distiller set	1
Soxhlet apparatus	5
Water bath	5
Thermolyne heating mantles, sizes 50, 100, 200, 1,000 ml	4
Vacuum pump	2
Micro melting point apparatus (Thomas-Hoover capillary melting point apparatus models 6406K and 6406H)	1
Autoclave	1
Magnetic stirrer and heater	2
Microscope	2
Microscope, polarized	1
KBr pellet maker and presser	1
Aspirator, motorized	5
Water aspirator set	10

Cool Ace cooler	2
Stirrer bars, many sizes	20
Column (glass), large, medium, and small sizes	20
Separatory funnel, many sizes	
Pressure equalizing funnel, various sizes	
Heavy duty mixer and blender	1
Water purifier for ultra pure	1
Incubator/shaker	1
pH meter	1
Cool room storage	1
Gas chromatograph (GC) + accessories (Flame Ionization Detector, Thermal Conductivity Detector, Electron Capture Detector)	1 set
Gas tuber for N_2, H_2, He, Ar, and Mannometer 4	
Air pump, automatic for GC	1
Chromatopack for GC recorder and processor	1
High-performance liquid chromatograph (HPLC), gradient, cooler, and heater	1
Miscellaneous columns for HPLC	
HPLC column packer and column supports	
Empty column for GLC	
Thin-layer chromatography package	10
Column chromatography package	10
Ion-exchange chromatography package	1
Empty glass column for GLC	
Infrared spectrometer	1
Automatic polarimeter	1
HPLC scanner	1
UV-Vis spectrophotometer	1
Personal computer and printer	1
Tape recorder	1
Camera and accessories	1
Glass vessel and apparatus	1
NMR 100 MHz spectrometer with ^{13}C and ^{1}H	1
Dreiding stereomodels (Rinco Instruments Co.)	1 set

<u>Items listed in the 1986-1987 Aldrich catalog</u>:

Adapters (pp. 1584-1588)	50
Pyrex gas washing bottle (pp. 1606)	10
Gas condenser bottle (pp. 1606)	5
Condensers (pp. 1634-1636)	15
Desiccators (pp. 1639-1640)	10
Drying units (pp. 1641-1642)	3
Distillation equipment (pp. 1645-1649)	3
Aldrich All-in-One Glassware kits (p. 1649)	2
Filter funnels (pp. 1653-1656)	25-50
Variable-speed motor	10
Variable transformer	16
Extraction thimbles (p. 1663)	100

Flash-vacuum thermolysis (p. 1664)	1 set
Dewar flasks (p. 1674)	10
Flow meters (p. 1676)	3
Flow indicators (p. 1677)	10
Gas accessories (pp. 1683-1686)	10
Gauges (p. 1687)	10
Diazald Kit for the safe preparation of diazomethane (p. 1716)	2
Corning Organic Chemistry Glassware kits (p. 1720)	1
Microscale organic laboratory glassware (p. 1721)	1 set
Lubricants for stirrer	10 jars
NMR sample tubes (p. 1728)	5
NMR tube cleaner (p. 1729)	
pH papers	
Photochemical equipment (p. 1734)	1 set
Eye protectors (p. 1747)	
Protective clothing (p. 1748)	
Stirrer blades, Teflon (p. 1758)	20
Stirrer bearings and shafts (pp. 1758-1759)	20
Stoppers (pp. 1760-1761)	100
Sublimation equipment (p. 1763)	1
Clamps	50
Lattice support systems (pp 1764-1768)	10
Lab jacks (p. 1770)	25
Thermometer with ground joints (pp. 1782-1783)	10
Volumetric ware, cylinders (pp. 1806-1812)	100
Parr hydrogenation apparatus (both low and high pressure)	2
Thermometer	10
Snap-on all-purpose lab tool set, 95 pieces (Z14,864-4)	1 set

PART III

Working Group Papers

SITOSTEROL SOURCES FROM AGRICULTURAL BY-PRODUCTS AND NATURAL RESOURCES

Status of Raw Materials for the Production of Oral Contraceptives in Indonesia

Monroe E. Wall
Chemistry and Life Sciences Division Group,
Research Triangle Institute

INTRODUCTION

This paper describes the current worldwide status of a variety of steroid sources for the production of steroid hormones, and it relates this information to the status of any sources of steroids that may be available in Indonesia now or in the future.

About 35 years ago the U.S. Department of Agriculture (USDA), with special congressional funding, initiated a program that was somewhat similar to that now under way in Indonesia. The United States wished to establish sources of steroids for the production, initially, of cortisone, and later of contraceptive steroids, independent of foreign sources. This author was in charge of the chemical studies and worked closely with botanists and agronomists in the USDA Plant Introduction Group. Over 6,000 plants were investigated. Some of this experience, including some of the mistakes, may be useful even at this time, some 35 years later. Thus, much of the following is based on the experience gained in the USDA program.

RAW MATERIALS

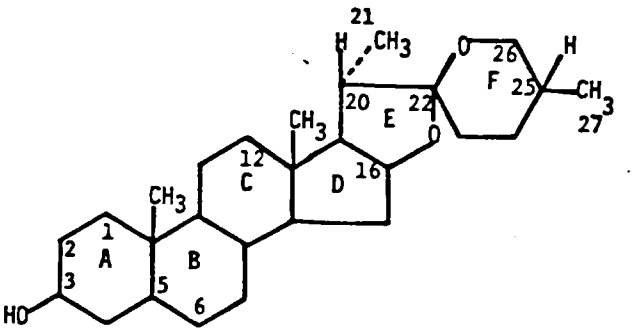
Steroid Sapogenins

These compounds are almost always found in nature in the form of steroid conjugates in which the steroid (usually at the 3-hydroxyl position) is linked to a number of sugar molecules. The free sapogenin is usually liberated by acidic hydrolysis (Wall et al., 1952), but plant or fungal enzymes can also carry out the hydrolysis (Krider and Wall, 1952; Krider et al., 1954). Indeed, this is often the way hecogenin is obtained from sisal juice.

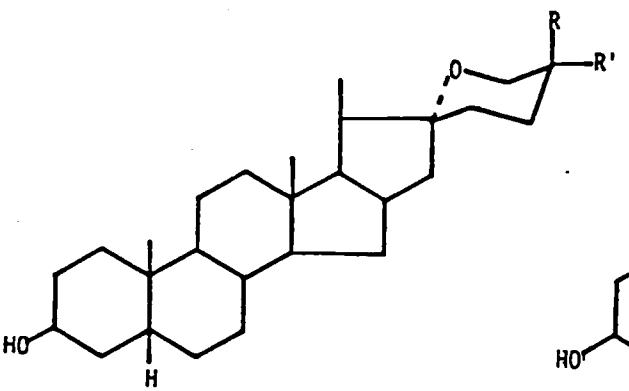
The structure of sapogenins and the plants from which they are derived are shown in Figures 1-3 and Table 1. Hecogenin and diosgenin are the only steroid sapogenins in commercial production at this time.

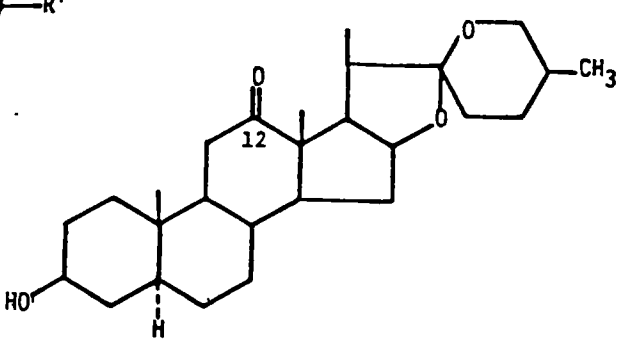
Hecogenin

In an extensive survey, hecogenin was found in many *Agave* species growing in arid regions of northern Mexico and the southwestern United



Typical Structure of Steroidal Sapogenins





I, R = CH_3, R' = H, Sarsasapogenin

II, R = H, R' = CH_3, Smilagenin

Hecogenin

FIGURE 1 Typical structure of steroid sapogenins.

States (Arizona and New Mexico). Hecogenin was usually the predominant sapogenin, at times mixed with its 12-desoxy analogue tigogenin.

Agave sisalana, a plant with thick, long (3-4 feet) leaves grown for fiber, contains various concentrations of hecogenin. In our experience the sapogenin content was usually low, 0.1-0.2 percent MFB (moisture-free basis). During fiber production, however, a huge volume of juices and wash liquids are obtained. In a large sisal operation observed by this author many years ago in Haiti, such liquids were held in huge tanks, and an insoluble sludge containing hecogenin could be

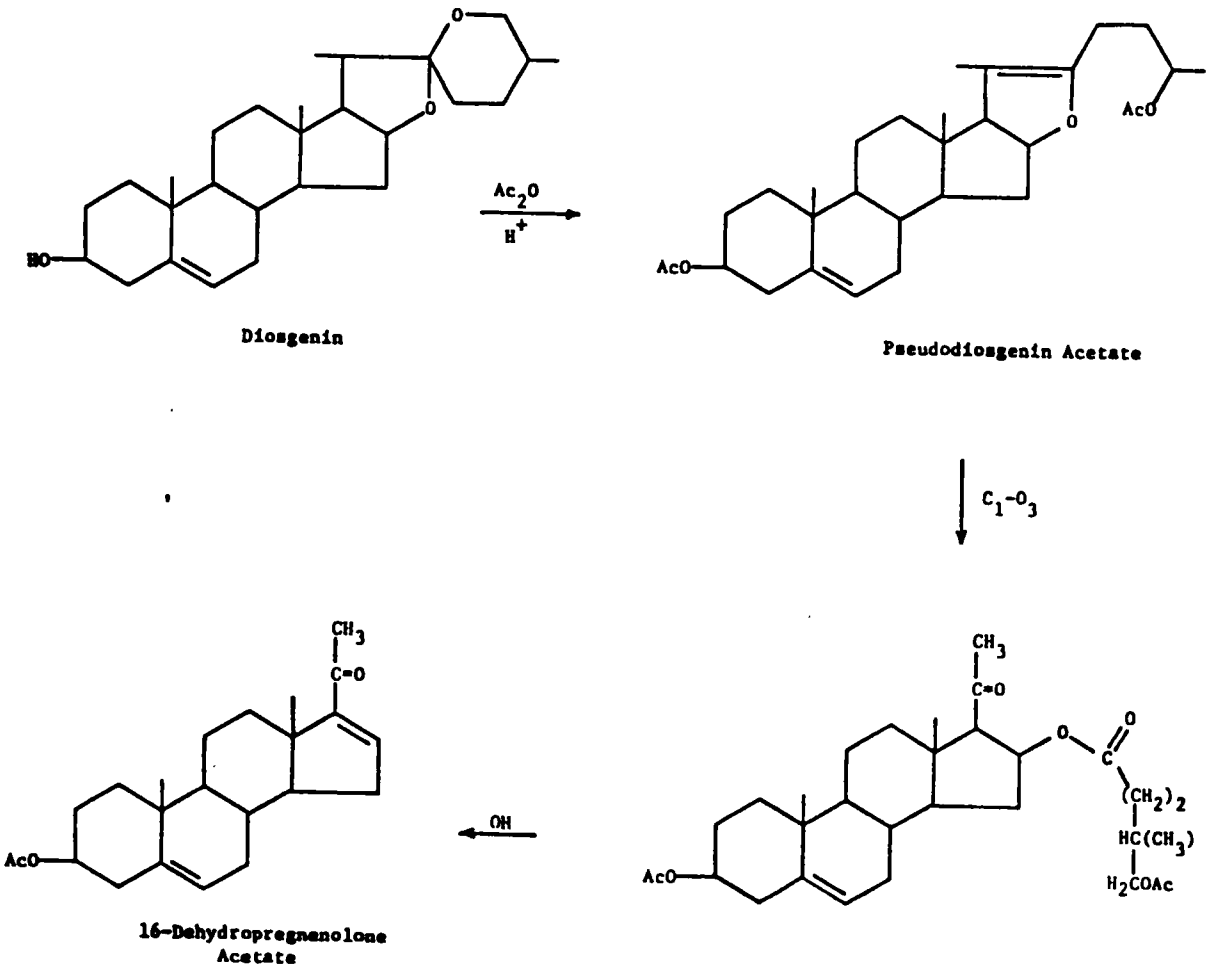


FIGURE 2 Degradation of diosgenin via pseudodiosgenin acetate to 16-dehydropregnenolone acetate.

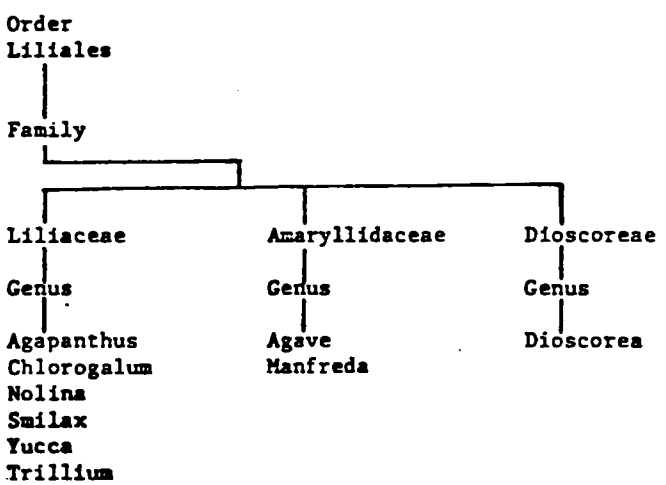


FIGURE 3 Botanical relationships of sapogenaceous plants.

TABLE 1 Steroid Sapogenins and Plant Sources

Name	A/B Fusion	Isomerism at C25	Substituents	Typical Plant Source
Sarsasapogenin	β	neo	3β-OH	Y. Baccata
Smilogenin	β	iso	3β-OH	A. lecheguilla
Markogenin	β	neo	2β, 3β-(OH)₂	Y. schidigera
Samogenin	β	iso	2β, 3β-(OH)₂	Y. carnerosana
Willagenin	β	neo	3β-OH, 12 CO	Y. filifera?
Mexogenin	β	iso	2β, 3β-(OH)₂, 12 CO	Y. schottii
Neotigogenin	α	neo	3β-OH	A. sisalana
Tigogenin	α	iso	3β-OH	Y. peninsularis
Gitogenin	α	iso	2α, 3β-(OH)₂	A. schottii
Sisalagenin	α	neo	3β-OH, 12 CO	A. sisalana
Hecogenin	α	iso	3β-OH, 12 CO	A. sisalana
Manogenin	α	iso	2α, 3β-(OH)₂, 12 CO	A. nelsonii
delta⁹-Hecogenin	α	iso	3β-OH, $\Delta^{9(11)}$, 12 CO	A. nelsonii
Chlorogenin	α	iso	3β, 6α-(OH)₂	Chlorogalum pomeridianum
Digitogenin	α	iso	2α, 3β-(OH)₂	Digitalis purpurea
Yamogenin	Δ^5	neo	3β-OH	D. bartlettii
Diosgenin	Δ^5	iso	3β-OH	D. composita
Yuccagenin	Δ^5	iso	2α, 3β-(OH)₂	Y. filamentosa
Kammogenin	Δ^5	iso	2α, 3β-(OH)₂, 12 CO	Y. filamentosa
Centrogenin	Δ^5	iso	3β-(OH), 12 CO	D. spiculiflora
Correllogenin	Δ^5	neo	3β-(OH), 12 CO	D. spiculiflora

obtained from the enzymatic hydrolysis of water-soluble saponins to insoluble sapogenins. The solids were recovered by centrifugation.

Only one company, Glaxo in England, utilizes hecogenin, which is obtained as a by-product of sisal fiber production in East Africa. The sapogenin is converted to a cortisone analogue in a complex, multistep synthesis.

Hecogenin is not useful for the production of contraceptives, however, and commercial sisal production in Indonesia is limited.

Sarsaspogenin and Smilagenin

Sarsaspogenin is found in many Yucca species, which grow in the same generally arid habitats of northern Mexico and the southwestern United States (almost identical smilagenin is found in Agave lecheguilla in Texas). At one time the Indians native to these areas used the plants for detergents and for fiber.

Although the sapogenin content of the various yucca leaves is not high, rarely exceeding 1.0 percent MFB, this author found very high concentrations of saponin in the seeds—from 6 to 12 percent (Table 2). This is indeed a reasonable starting point for steroid hormone production, if sufficient plant volume is available.

Sarsasapogenin was readily extracted in sizable quantities and converted to 16-dehydro-5 -pregnenolone (Table 3). Although the process was a technical success, a profitable price for the sapogenin or its 16-dehydro-degradation product could not be obtained from pharmaceutical companies. Similar 16-dehydro steroids from smilagenin found in Agave lecheguilla also turned out to be not viable economically. The lesson to be learned is that starting materials such as sapogenins, or intermediates such as the 16-dehydropregnenes,

TABLE 2 Sapogenins Obtained from Seed Saponins

Species	Location	Sapogenin, % MFB
Yucca arizonica	Southern Arizona	Sarsasapogenin, 12.0
Yucca baccata	Superior, Arizona	Sarsasapogenin, 6.8
Yucca brevifolia	St. George, Utah	Tigogenin, 8.0
Yucca mohavensis	San Diego, Calif.	Sarsasapogenin, 6.6
Yucca schotii	Fort Huachuca, Ariz.	Sarsasapogenin, 4.9
Yucca spp.	Southern Arizona	Sarsasapogenin, 4.5
Yucca spp.	Sonora, Mexico	Sarsasapogenin, 6.2
Yucca spp.	Chihuahua, Mexico	Sarsasapogenin, 7.9

TABLE 3 Key Features and Plant Sources of Sapogenins from Which 16D is Derived

Sapogenin from Which 16D is Derived	Key Features	Plant Sources
Disogenin, yamogenin	Δ^5	*Dioscorea macrostachya*, *Compositae floribunda*
Gentrogenin, correllogenin	Δ^5, 12-keto	*D. spiculiflora*
Hecogenin	5α, 12-keto	Many *Agave*, *A. sisalana*
Smilagenein	5β	*A. lechequilla*
Sarsasapogenin	5β	Many *Yucca*, *Y. schidigera*
Tigogenin	5α	Many *Yucca*, some *Agave*, *Y. peninsularis*
Willagenin	5β, 12-keto	*Yucca filifera*

cannot normally be sold at profitable prices. It is necessary to proceed to production of the final useful steroid hormonal drugs.

Diosgenin and Solasodine

The pioneering efforts of Russell Marker almost 50 years ago resulted in diosgenin revolutionizing the steroid hormone industry. Diosgenin, which is found worldwide including Indonesia, occurs as a saponin, dioscin, in the tubers or the rhizomes of many Dioscorea species. In Mexico, tubers growing wild and of uncertain age have been found to contain 5-8 percent diosgenin (MFB). The diosgenin content of Indonesian Dioscorea is usually much lower. The rhizomes of various species of Costus, which grow well in Indonesia, have been found to contain from 1 to 3 percent diosgenin.

From whatever source, diosgenin has great versatility. Thus progesterone, and thereby certain orally active progesterone analogues such as Provera, can be derived from the readily obtained 16-dehydropregnenolone (Figure 4). Alternatively, the well-developed

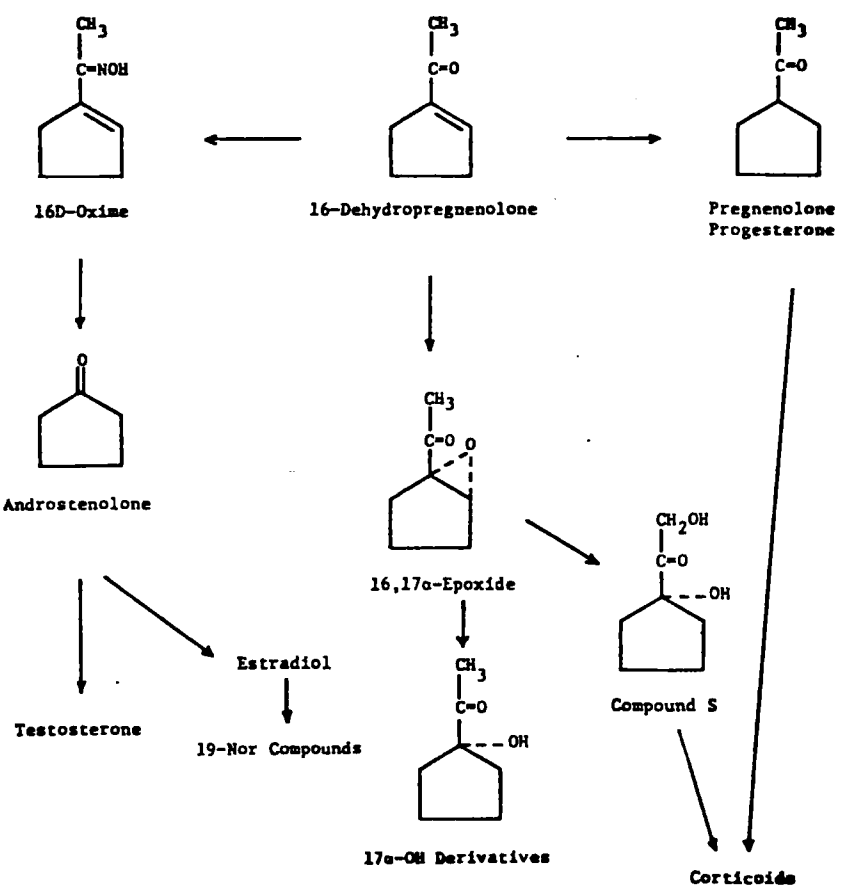


FIGURE 4 Structure of progesterone.

microbiological hydroxylation of progesterone leads to cortisone and many other useful corticosteroid hormones. In still another direction, 16-dehydropregnenolone, by a straightforward chemical route, can be converted to the useful C-19 steroid hormone precursor dehydroisoandrosterone (DHA). DHA can then be converted to the important 19-nor steroids and estrogens, which are components of many contraceptive drugs (Djerassi, 1976).

Solasodine is a steroid alkaloid found in a number of Solanum species that can be grown in Indonesia. Berries or fruits of S. khasianum and S. laciniatum have solasodine contents up to 4 percent. The chemistry of solasodine after side chain degradation is identical to that of diosgenin (Figure 5).

Plant Sterols

Until the early 1970s, diosgenin from Mexican sources dominated a large sector of the steroid hormone market. Restrictive practices by the Mexican government, however, led to a search for other sources.

In the United States, enormous quantities of soybeans are processed for the oil and residual meal. Soybean oil contains about 0.34-0.38 percent total sterols of which sitosterol constitutes about 53 percent and stigmasterol 23-24 percent (Gutfinger and Letan, 1974). Several billion bushels of soybeans are produced each year in the United States, and almost all the soybean crop is processed for oil. A sterol mixture is one of the by-products of a treatment normally used to refine crude soybean oil. This mixture is sold commercially and can be further processed by a complex countercurrent solvent treatment to yield 95 percent pure stigmasterol and 80 percent pure sitosterol, suitable for further fermentation to C-19 steroids.

Other sources of sitosterol include tall oil, a by-product of paper mills using pinewood, sugarcane wastes or oil, rice oil, and palm oil. None are in commercial use, nor have the processes for obtaining the sterol economically been worked out in detail.

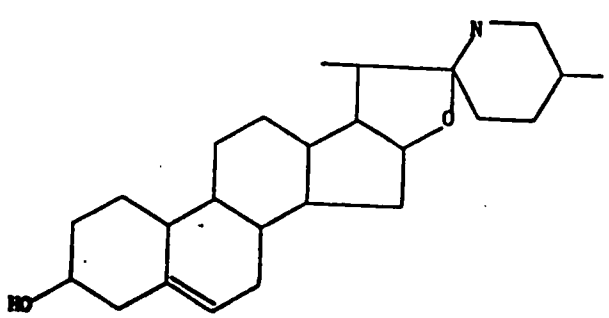


FIGURE 5 Structure of solasodine.

Stigmasterol

It has been claimed that 400,000 kg of stigmasterol are available from U.S. sources. This steroid is readily converted to progesterone (Figure 6), and compares favorably with the process using diosgenin. Unless soybean oil is available on a large scale, stigmasterol cannot be considered for Indonesian use. Perhaps the crude sterol mixture could be purchased cheaply as a starting material. Stigmasterol is one of the major steroids used by the Upjohn Company for synthesis of progesterone and thus corticosteroids and the oral contraceptive Provera.

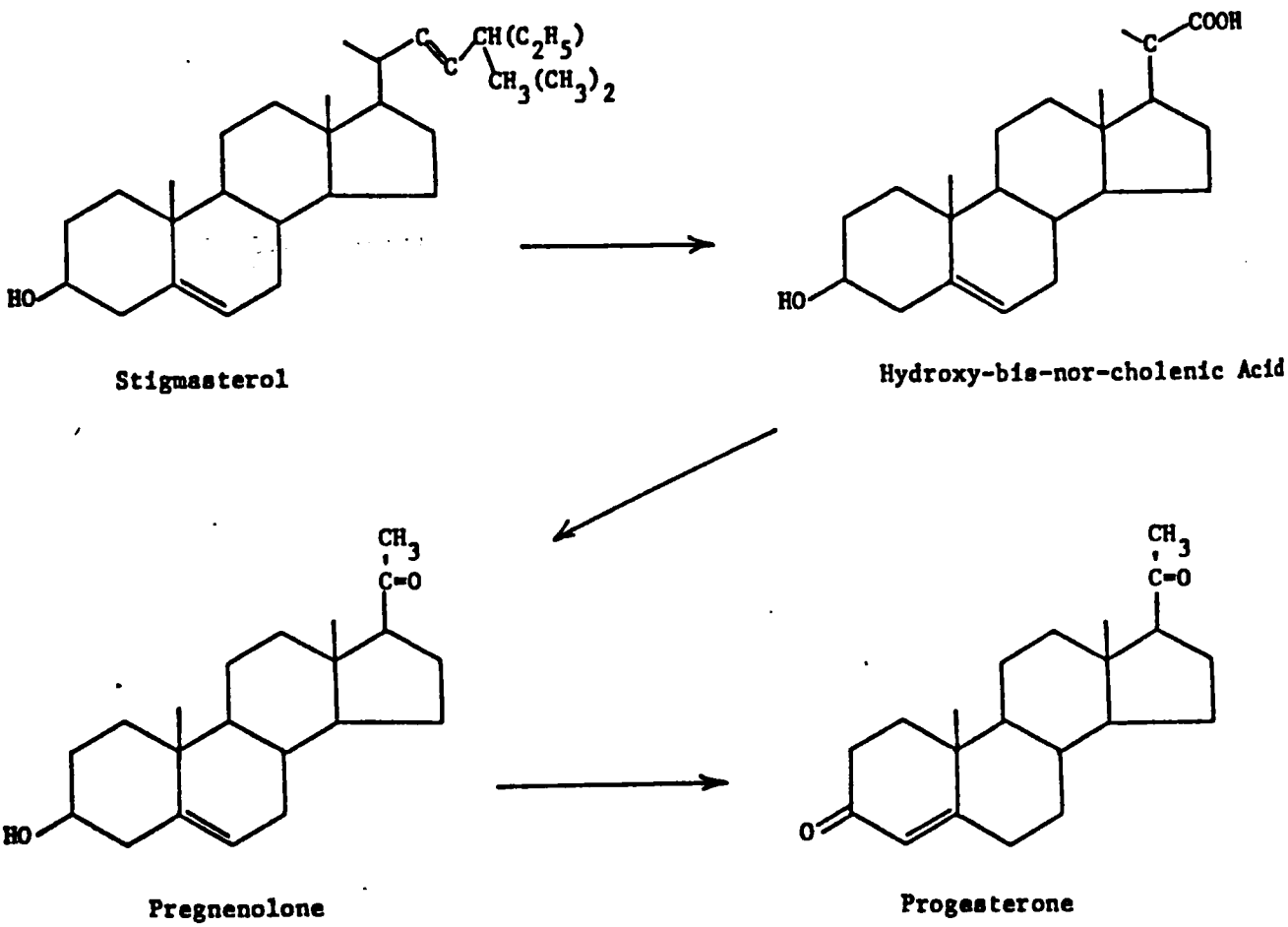


FIGURE 6 Conversion of stigmasterol to progesterone.

Sitosterol

Sitosterol is an ubiquitous plant sterol found in virtually all plants and plant parts. Until the late 1960s and early 1970s, this sterol and the equally ubiquitous animal sterol cholesterol could not be used for steroid hormone production. Almost simultaneously various U.S. patents (Kraychy et al., 1972) and a paper (Marsheck et al., 1972) appeared on converting sitosterol or cholesterol as well as other sterols to androsta-1, 4-diene-3, 17-dione in good yields using a particular strain of Mycobacterium or, alternatively, another Mycobacterium strain to form androst-4-ene-3, 17-dione (Figure 7). As will be shown in later presentations, these compounds are versatile and easily convertible to 19-nor steroids or ring A aromatic steroids. Thus, they are excellent sources for contraceptive drugs. Aldosterone, a steroid diuretic, can also be prepared from such sources (Djerassi, 1976).

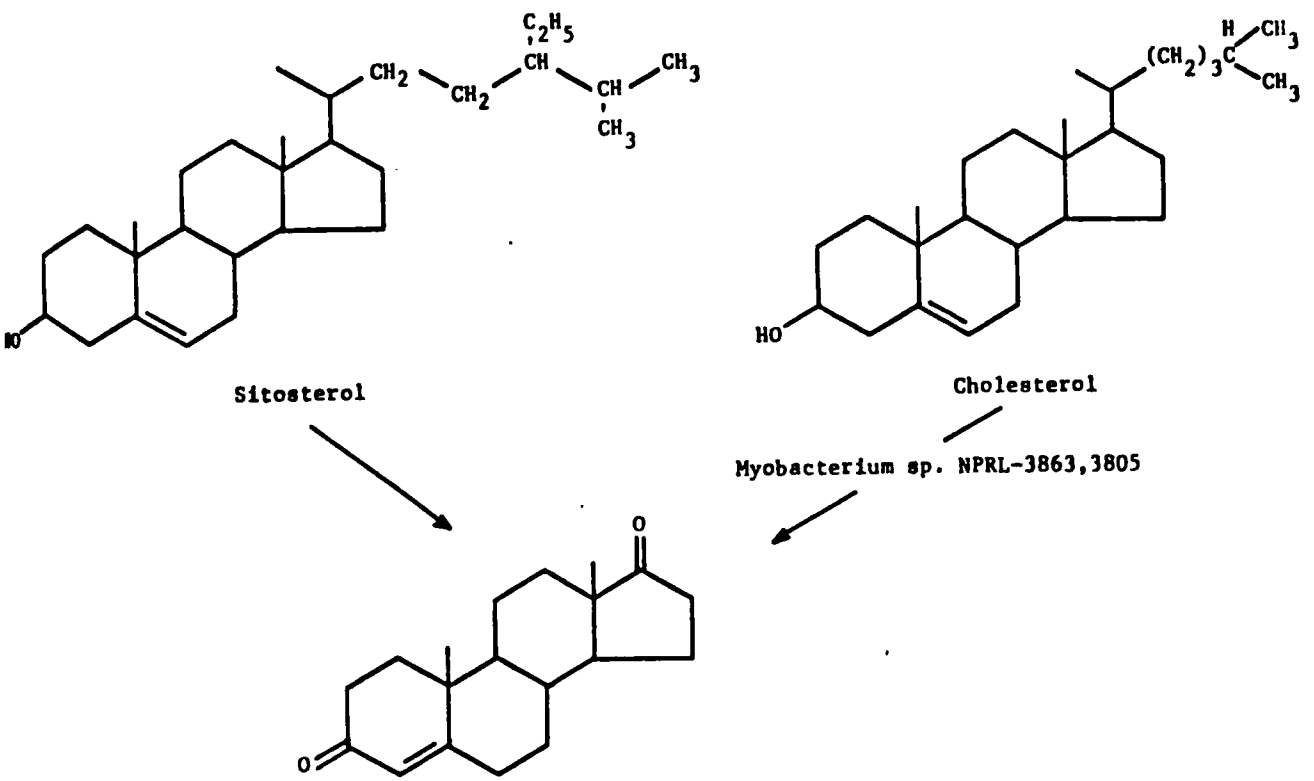


FIGURE 7 Microbiological conversion of sitosterol to androst-4-en-3-one.

FUTURE PLANS

Which steroids and plant sources should be selected for further study in Indonesia, leading to the production of contraceptive drugs? It is evident from previous studies that the sapogenin content of most plants currently found in Indonesia may be too low for economic production of the starting steroid. Problems with cultivation, such as disease or thorns in the case of Solanum, have also been encountered.

On a long-range basis, the marked advances in biotechnology, which are appearing with increasing rapidity, may be useful. For example, if the genetic pattern of Dioscorea could be altered to obtain high-yielding, disease-resistant strains that give yields of at least 5 percent in two years, economic production might be possible. On the other hand, a high yield could perhaps be obtained by cell culture. Similar questions apply to the Costus and Solanum species. While advances in biotechnology make such suggestions less wild than they would have been 30 years ago, implementation of these suggestions would still require at least 5-10 years.

It is also time to begin large laboratory-scale, and then pilot-scale, studies of the conversion of diosgenin or solasodine to 16-dehydropregnenolone and thereby to androst-4-ene-3, 17-dione. For this purpose, sufficient starting material for the isolation of diosgenin could be collected or purchased. For sitosterol and cholesterol, Indonesian scientists could commence fermentation studies. Both of these areas, which should be pursued intensively, will require trained chemists and microbiologists.

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Potential Natural Sources of Sitosterol

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INTRODUCTION

Early steroid production relied initially on the extraction of natural hormones from animal sources and, later, on partial synthesis from cholesterol. It was not until the 1940s, when Russell Marker showed that steroid sapogenins obtained from plants, in particular diosgenin, were well suited to the preparation of certain hormones, that a cheaper raw material emerged. In 1944, Syntex, the company founded by Marker, began the production of progesterone from Mexican sources of diosgenin. At that time there was still no large market for sex hormones, but in 1949 the action of cortisone in suppressing the symptoms of rheumatoid arthritis was discovered, thus requiring the production of large amounts of cortisone.

In the late 1950s, techniques were developed that eventually allowed stigmasterol, a component of soybean oil, to be utilized. Finally, the further development of microbiological methods resulted in the utilization of sitosterol in 1976 and the reemergence of cholesterol in 1978 as precursors (Coppen, 1979). This paper describes potential natural sources of sitosterol.

PRECURSORS FOR PARTIAL STEROID SYNTHESIS

Most steroid drugs are made by partial synthesis from suitable, naturally occurring precursors. In 1944, progesterone was produced from diosgenin. Stigmasterol from soybean oil is used for the production of corticosteroids, as are hecogenin from *Agave* and bile acids from animal sources. Recent developments in the steroid industry have led to the commercialization of certain microbiological processes that now enable such precursors as sitosterol from soybean and cholesterol from wool grease to be used.

The main precursors used in the production of steroid drugs are shown in Figure 1, and examples of routes to the corticosteroids and oral contraceptives are given in Figures 2 and 3.

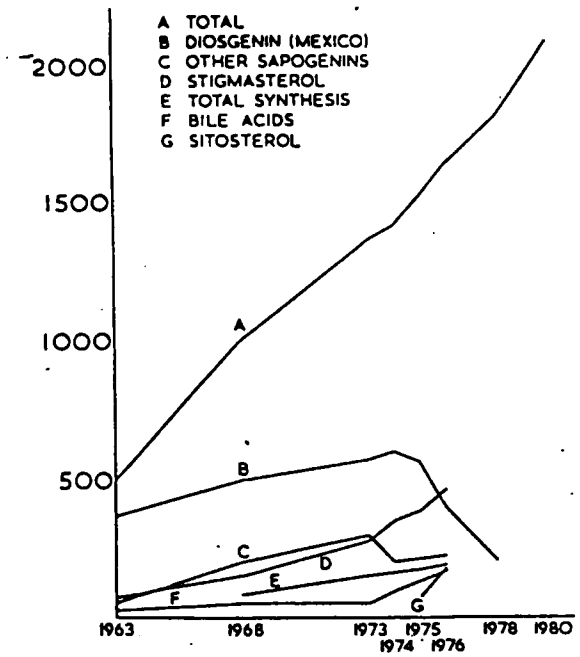

FIGURE 1 Production of steroids (tons diosgenin equivalent). Reemergence of cholesterol in 1978 is not shown.

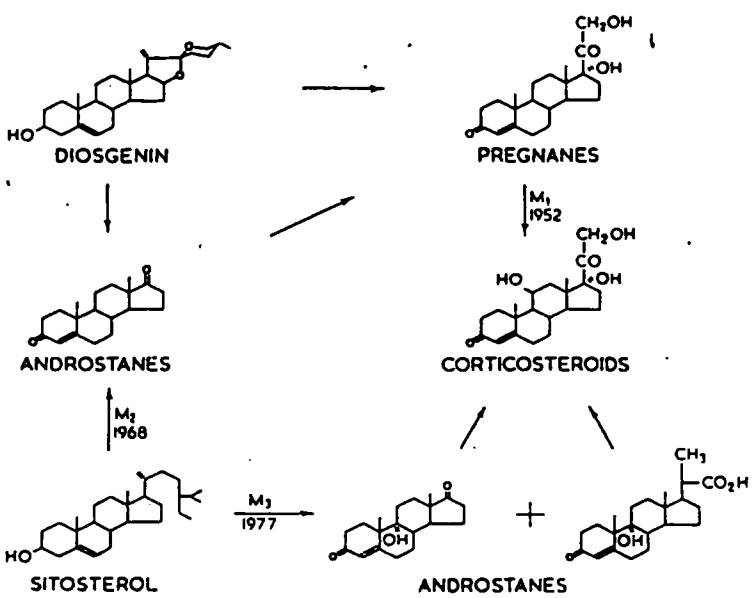

FIGURE 2 Routes to the corticosteroids.

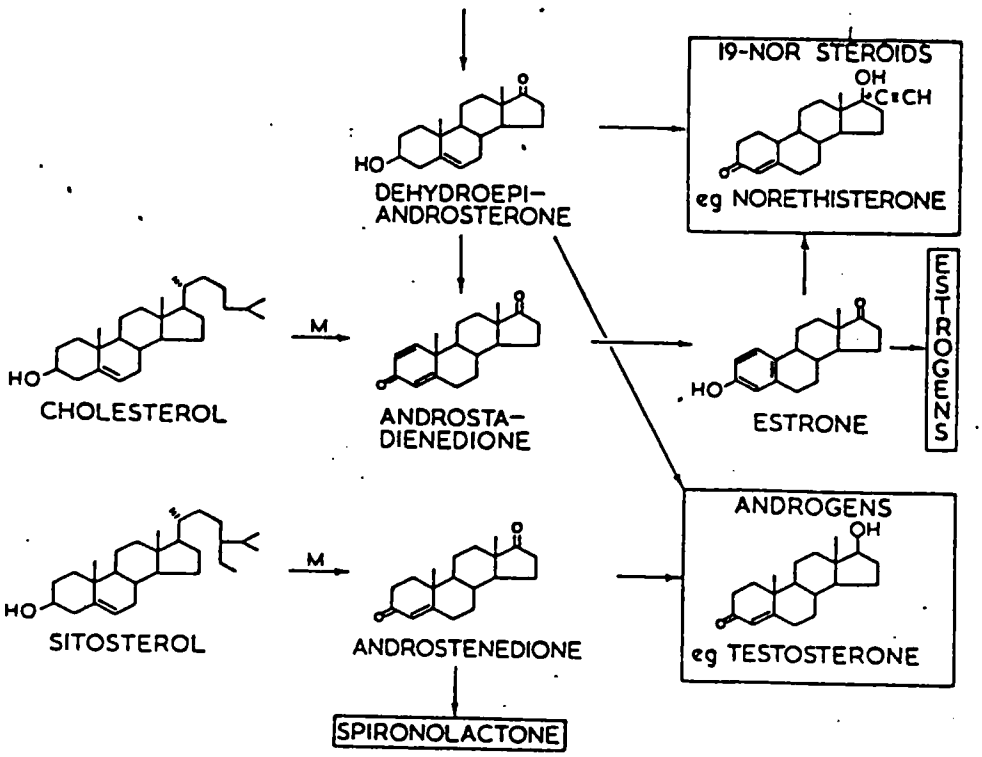

FIGURE 3 Main routes from sterols to products.

STEROLS

Sterols, the most widely distributed group of steroids in the plant kingdom (including algae, fungi, and higher plants) commonly occur as mixtures of sitosterol, campesterol, stigmasterol, and other sterols. The exact composition depends on the source. Any utilization of sitosterol or stigmasterol requires therefore a readily available source and a means of separating the desired sterol from the other sterols present. The increasing contribution of the sterols to the production of steroid drugs and contraceptives is shown in Figure 1.

Sterols as a By-product of Industry

Sitosterol and stigmasterol, utilized in the present production of steroids, are by-products of soybean oil processing. These sterols are contained in the nonsaponifiable matter of the oil. Most fatty oils contain 0.1-2.0 percent nonsaponifiable matter.

The major oils and fats produced worldwide are shown in Figure 4, and the nonsaponifiable contents are given in Table 1. The production of soybean oil will have doubled by the year 2003, although its share

will remain 22 percent of total oil production. Palm oil production will increase from an annual production of 4.5 million tons for the period 1978-1982 to about 21 million tons for the period 2003-2007. The production of other oils will also increase. These oils, whose by-products are not utilized, should be considered.

Nonsaponifiable matter contains various sterols with different compositions, but of those sterols present only sitosterol and stigmasterol are utilized after they are separated using countercurrent

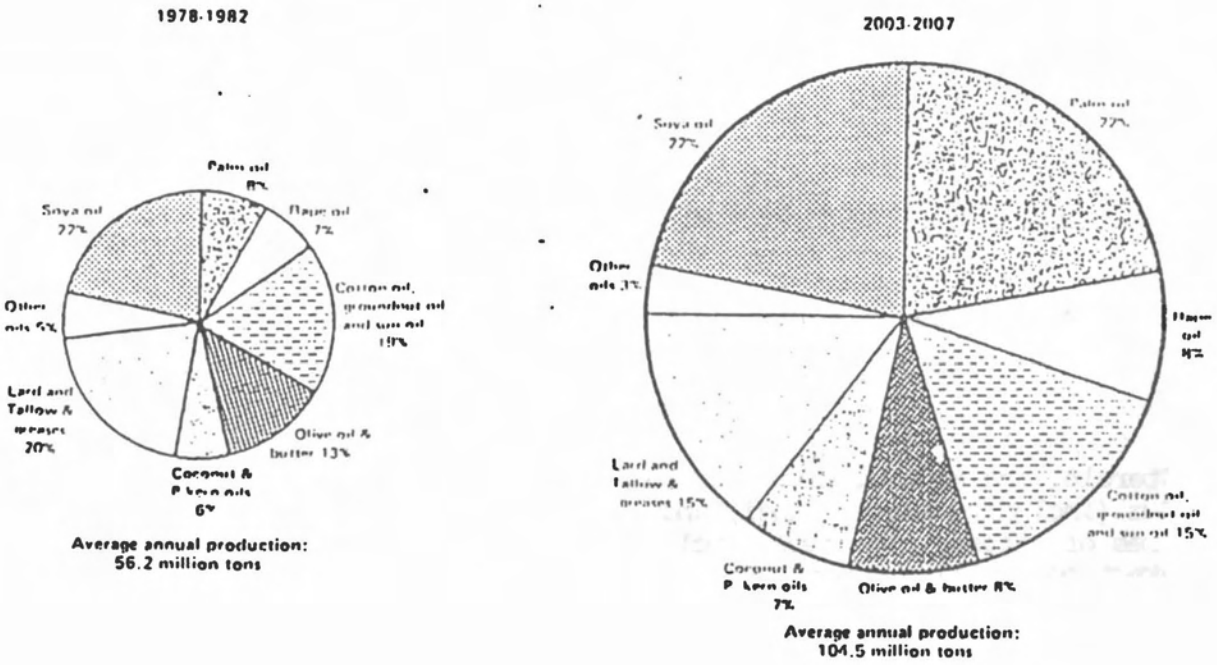

FIGURE 4 Shares of total production (percent) of the major oils and fats, 1978-1982 and projection for 2003-2007.

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TABLE 1 Percent of Nonsaponifiable Matter of the Major Oils

Oil	Plant	Nonsaponifiable Matter (percent)
Coconut	*Cocos nucifera*	0.2
Peanut	*Arachis hypogaea*	0.5-0.9
Palm	*Elaeis guineensis*	
Corn	*Zea mays*	1.5-2.8
Rapeseed	*Brassica*	1.5
Soybean	*Gycine max*	1.3-1.5
Cottonseed	*Gossypium hirsutum*	1.1
Sunflower	*Helianthus*	0.3
Almond	*Prunus amygdalus*	0.75
Olive	*Olea europaea*	0.4-1.0
Sesame	*Sesamum indicum*	0.9-1.3

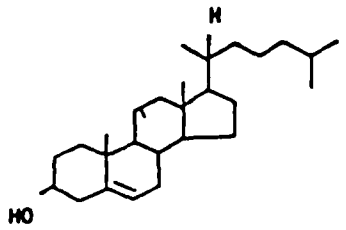
crystallization. Figure 5 shows the composition of soybean sterols, and the isolation scheme of soybean sterols is given in Figure 6. This isolation method could be adapted to separation of the sitosterol contained in other oils.

Tall oil, a by-product of the pulp industry, also contains sterols. It has not been utilized because it is found in relatively small quantities. Yet another source of sterols is pressmud from the sugarcane industry.

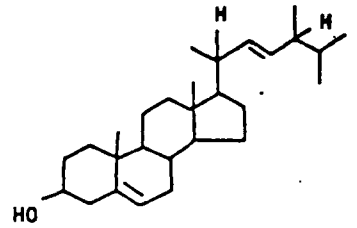
Sterols from Higher Plants

The sterol contents of various plants have been published. One example is the sterol composition of the seeds and mature plants of the Cucurbitaceae family (Akihisa et al., 1986).

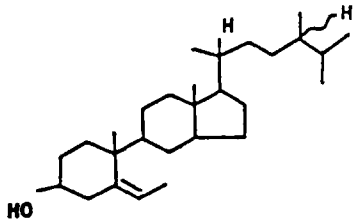
Criteria for the evaluation of these sources of sterols—such as technical feasibility, availability in Indonesia and abroad, universality, and economical feasibility—must be established.



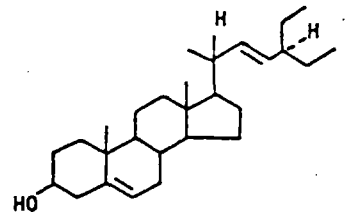
Cholesterol (trace)



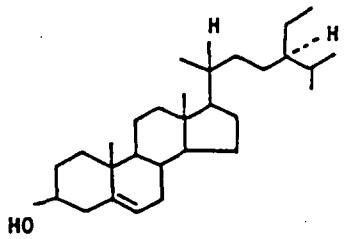
Brassicasterol (trace)



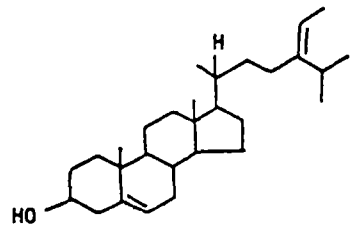
"Campesterol" (20%)



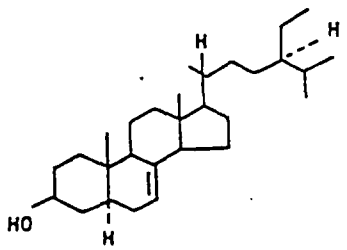
Stigmasterol (20%)



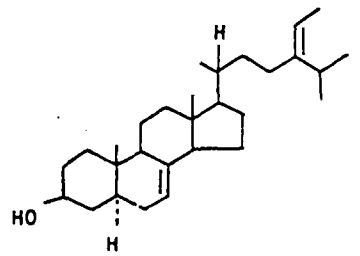
Sitosterol (53%)



Δ^5 - Avenasterol (3%)



Δ^7 - stigmasterol (3%)



Δ^7 - Avenasterol (1%)

FIGURE 5 Composition of soybean sterols.

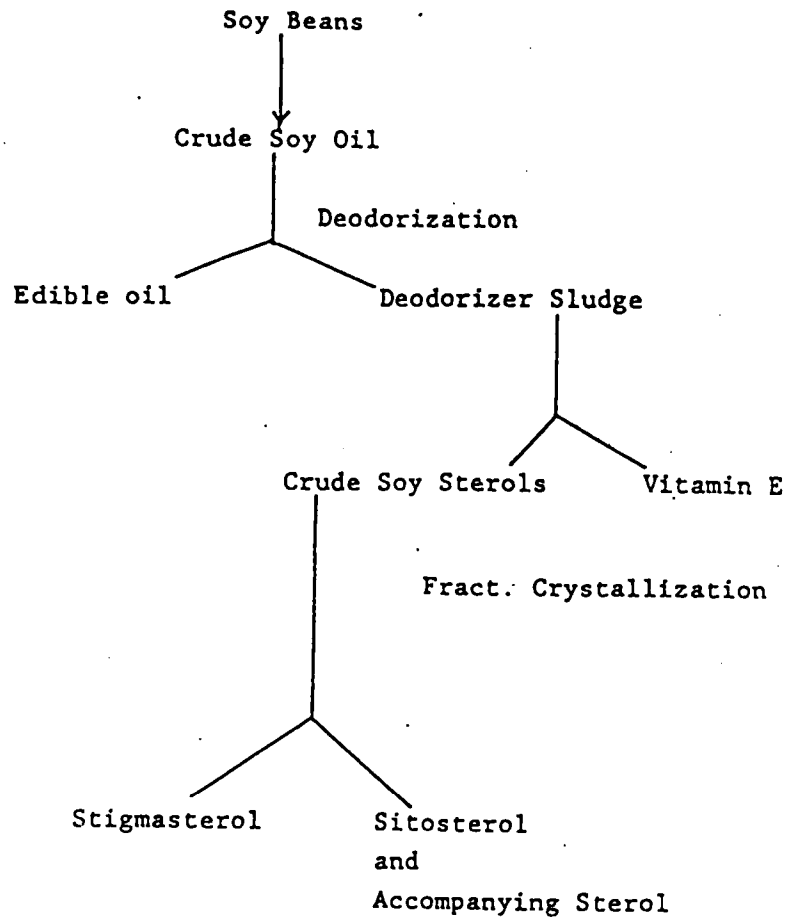


FIGURE 6 Isolation scheme of soybean sterols.

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PRODUCTION OF STEROID COMPOUNDS BY PLANT CELL AND TISSUE CULTURE

The Role of Plant Tissue Culture in Pharmacy and Biotechnology

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Convincing evidence exists that 50 percent or more of our drugs originate from microbial products and the higher plants. Unfortunately, many in the technologically advanced countries believe natural product research to be unimportant (Staba, 1985).

Plant tissue culture (PTC) refers to the in vitro cultivation of any plant part, whether a single cell, a plant tissue or organ, or an entire plant, under aseptic conditions. The technique is sometimes used to replicate and micropropagate plants more efficiently for field cultivation. It may also be used to develop new plants with improved qualities, such as disease or herbicide resistance or an ability to grow in brackish water, and to improve a plant's chemical composition. Simply put, the objectives are to improve the time required to grow plants and to improve them for field cultivation.

Micropropagation is used routinely and profitably in the ornamental industry to produce large numbers of orchids, carnations, and ferns; in the lumber industry to reforest land with eucalyptus and pine; and in the specialty crop business in Malaysia to produce palm oil, in South America to produce pyrethrum, and in India to produce glycyrrhiza. How this is accomplished by the PTC technique is briefly summarized in Figure 1.

The PTC technique is also used in the in vitro production of biochemicals, with the aim of eliminating the need to use cultivated plants to obtain expensive biochemicals and the need for special climatic conditions. To date, only two commercially successful PTC systems have been developed for this purpose--both in Japan. These systems produce an expensive cosmetic dye, shikonin, and ginseng ginstnosides.

It is important to recognize that many types of plant tissue culture are considerably more complex and difficult to grow than microorganisms. To undertake plant tissue culture, a part of the plant is explanted and grown aseptically on a simple, defined agar medium that contains small concentrations of growth regulators such as 2,4-dichlorophenoxyacetic acid. If conditions are favorable, the explant will form a mass of unorganized cells known as callus. These cells may be undifferentiated; in many ways they resemble tumor cells. If these cells are transferred to a liquid medium, they will

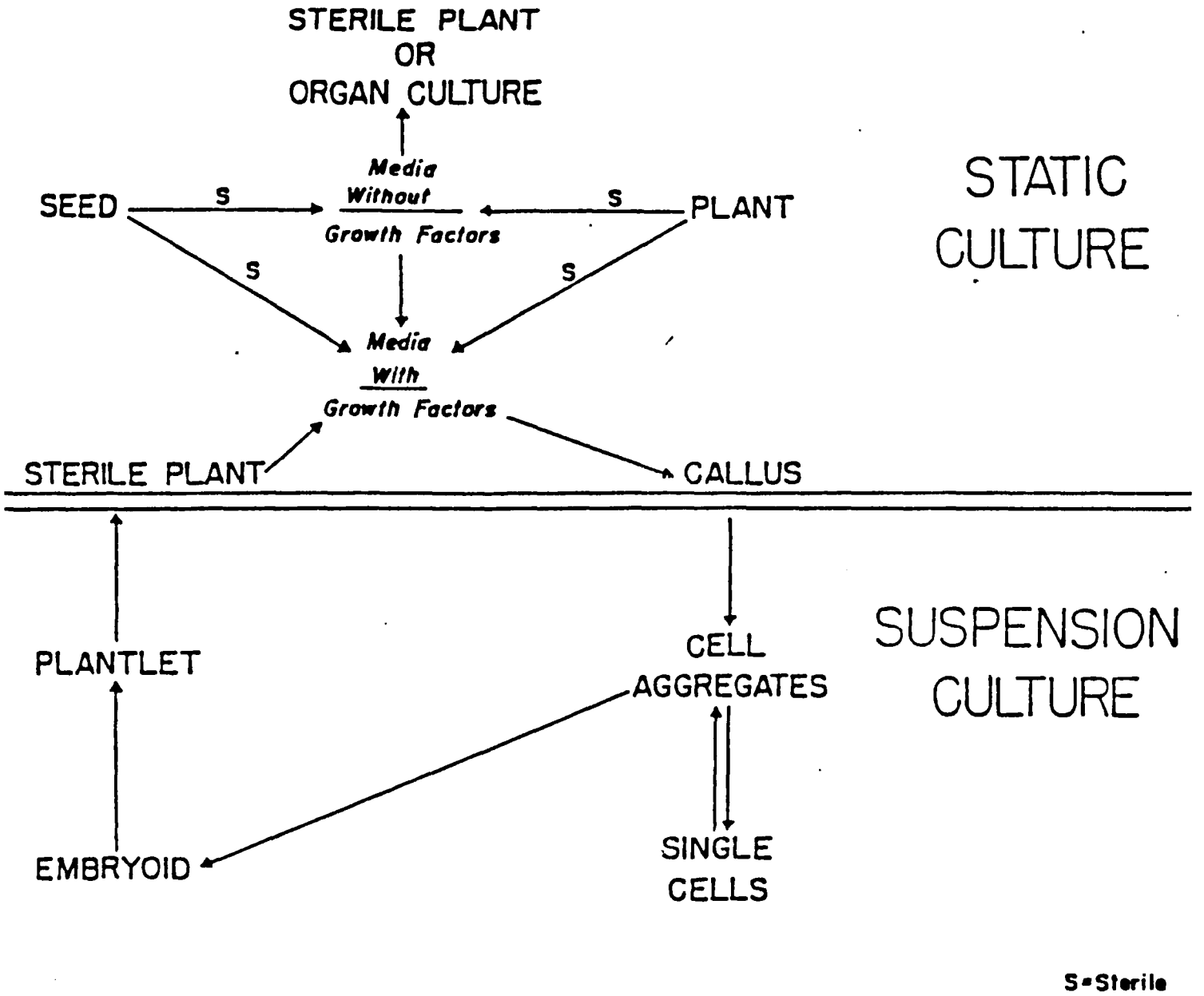

FIGURE 1 Plant tissue culture systems.

grow as either small or large cell aggregates. They may be subcultured every few weeks, and they are often maintained for indefinite periods of time. Aggregates of cells have been grown in commercial fermentors containing 20,000 l or more of medium. Such cell aggregates are often genetically and chemically variable, but they sometimes produce significant amounts of biochemicals such as rosemarinic acid (15 percent) and diosgenin (7 percent).

Plant organ cultures are more stable genetically than callus cultures, but they are more difficult to grow in large growth systems. Plant explants grown on a basal medium containing indoleacetic acid often form root cultures, while those grown on a medium containing benzyladenine form shoot cultures and those grown on a medium containing selected ratios of growth regulators form embryoids ("artificial" seeds) (Walker, 1986). Callus cultures can also form these same organ cultures when grown properly. Normal embryoids, root organs, shoot organs, callus, and even single plant cells can reform into a normal plantlet. The ability of a single plant cell to reform a normal plant without fertilization is known as totipotency. Organ cultures sometimes produce secondary products more efficiently than unorganized cells--for example, cardenolides from embryoids, pyrethrins from shoot cultures, and scopolamine from root cultures. Root organ cultures have been used to study alkaloid production for decades. As long ago as 1965, a patent was issued to the Merck Co. (West Germany) to produce secondary projects from root organ cultures. More recently, commercial concerns have been growing plant tissue cultures using the root-inducing microorganism Agrobacterium rhizogenes to produce secondary products. Shoot organ or meristem cultures are most often used for micropropagation processes. Embryoids coated with gels are being used in the United States for "artificial" celery seed production and in Japan for the commercial production of ginseng glycosides.

Special strategies are sometimes used with plant cell aggregates to increase their yields of biochemicals--for example, selection of cells or organs for high production rates; development of a biomass medium for maximum growth and a production medium for high yield (two-step process); use of elicitors for stress induction; and use of immobilized cell systems. Elicitors are nutritional, hormonal, toxic compounds or environmental changes that trigger increased production for reasons that are largely unknown (DiCosmo and Tower, 1984).

Traditional strategies involving control of the growth phase (most often the stationary phase), pH, precursors, and environment must also be studied and used where appropriate (Sahai and Knuth, 1985). The conversion of nonuseful into useful chemicals in plant tissue culture has also been studied. This is exemplified by the conversion of digitoxin into digoxin by digitalis cell aggregates.

It is possible to make protoplasts from aseptic explants of pollen, leaf mesophyll cells, and cell suspension aggregates. Protoplasts are plant cells whose walls have been dissolved by pectinase, cellulase, and hemicellulase enzymes. These cells are still living, however, and they will reform their cell walls in a number of hours. Nevertheless, while they exist as protoplasts they may be grown with Agrobacterium

tumefaciens microorganisms which bring into the cell important coded plasmid DNA for, perhaps, disease resistance, improved resistance to an unfavorable environment, increased product production, or new product production such as sweeteners, proteins, or antibiotics. Because the protoplast is totipotent, it can be regenerated into plants that, it is hoped, retain the new attribute as well as the desirable old attributes. Plasmid amplification, chromosome integration, and retention are desired. It is also possible to fuse two protoplasts from different plant species to produce a new, improved plant.

Protoplast technology is difficult. Interesting experimental results and plants have been obtained from genetic engineering or fusion studies, but none, except environmental selection from potato protoplasts, have been introduced commercially. The future of PTC must be viewed with optimism, however. As our knowledge of plant physiology and function increases in parallel with biological system development, we will see more successful commercial processes (Collin and Watts, 1985; Klausner, 1985).

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The Contribution of Plant Cell and Tissue Culture to the Production of Steroid Compounds in Indonesia

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The potential of plants to serve as a source of raw material for the industrial production of steroids is undeniable, despite the fact that many countries have abandoned this source. Dioscorea floribunda from Mexico was used for 20 years before it was replaced by other sources in 1974. This was attributed to an unstable supply and the agricultural situation in Mexico. Solanum laciniatum, another plant considered for steroid production, faced the same problems. In fact, in Hungary the project in this area was dropped given the climatic conditions there and the economic condition of Hungarian agriculture (Kovats and Richter, 1982).

Indonesia is rich in plant species, which are distributed over its many islands. Apart from Dioscorea and Solanum, Costus spp. have also been found to contain diosgenin. Lubis (1982) reported that the seeds of Costus speciosus contained 3 percent diosgenin and the tuber 1.2 percent. These percentages are still not adequate, however, when they are subjected to a cost-price analysis. Because manipulation of plant growth using the culture technique will not necessarily result in an improved secondary metabolites content, systematic breeding programs to improve the content of active material should be a priority.

Conventional breeding programs have technical problems and require generations to achieve a certain quality. An alternative approach is use of the plant cell and tissue culture method, which is more rapid through manipulation of cells and molecules. An in vitro breeding program should also aim to improve plant architecture and other agronomic characteristics suited to improving commercial plantations for Costus spp., Dioscorea spp., and Solanum spp.

Use of the plant cell and tissue culture technique in producing secondary metabolites may not look promising at the moment, but it will be beneficial over the long term. Secondary metabolite production can be increased manyfold by manipulating the culture medium component (PGR, precursor, nutrient), incubation environment, and cell growth and differentiation.

Based on the availability of plant species, their environmental adaptability, experience with plant cell and tissue culture, and available facilities, work on Costus spp. should rank first, followed

by work on Discorea spp. and Solanum spp. This ranking does not necessarily apply to tissue culture work on producing secondary metabolites.

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PRODUCTION OF STEROID COMPOUNDS BY FERMENTATION AND CHEMICAL SYNTHESIS

Synthesis of Steroid Oral Contraceptives Available in the United States

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INTRODUCTION

Scope

This presentation reviews and summarizes the best synthetic methodology of steroid oral contraceptives currently marketed in the United States. Reviews of steroid oral contraceptives were reported earlier by Briggs,[1] Colton and Klimstra,[2] Djerassi,[3] Drill,[4] Gold et al.,[5] Klimstra and Colton,[6] Payne and Green,[7] Petrow,[8] Pincus and Merrill,[9] Velluz,[10] and Windholz and Windholz.[11] Thus, this presentation will be confined to the most efficient partial synthesis of several 19-nor steroids, and the total synthesis of norgestrel, which is currently available in the United States, and two steroid estrogens, ethynylestradiol (EE) and mestranol (MEE).

Historical Development

The basis for the design of steroid contraceptive agents dates back to the beginning of this century when corpus luteum extracts injected into various animals were found to prevent ovulation. In 1934, the active component of the corpus luteum was isolated and its structure elucidated as pregn-4-ene-3, 20-dione (progesterone). Progesterone (I) is only slightly active, however, when administered orally. A more active, preferably orally active, product was therefore needed.[8] In 1944, Ehrenstein[12] at the University of Pennsylvania reported the multistage transformation of the cardiac aglycone strophanthidin (II) to an oily mixture of stereoisomers of "19-norprogesterone (III)" in a 0.07 percent yield (Figure 1). The purpose of this work was to remove the angular methyl group, C-19, attached to position 10 of progesterone, and to examine the effect of such a structural change on progestational activity.

The chemical steps involved isomerization of three asymmetric centers (positions 17, 14, and perhaps 10 in III). This appeared

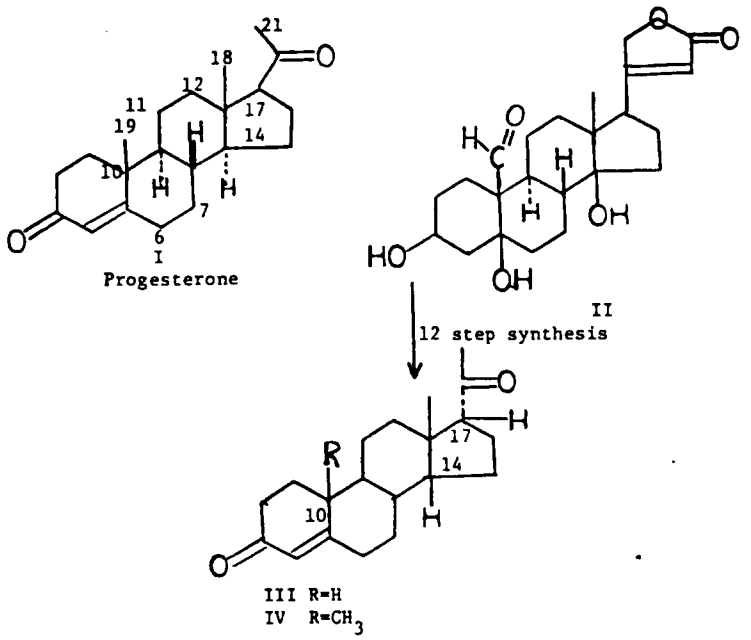

FIGURE 1 Ehrenstein's compound.

unfortunate, however, since it had been shown earlier that even inversion just at C-17 abolishes biological activity.[13] It was most surprising, therefore, to find that this oily material showed the same progestational activity as progesterone itself. Prompted by these unexpected biological results, a Swiss group[14] undertook the synthesis of 14-iso-17-isoprogesterone (IV), since its wrong stereochemistry at C-14 and C-17 mimicked that of Ehrenstein's compound (III) and it seemed conceivable that inversion of the stereochemistry of progesterone at both C-17 and C-14 could have been responsible for the biological activity of Ehrenstein's substance (III). The pure crystalline 14-iso-17-isoprogesterone (14β,α17-pregn-4-ene-3,20-dione) (IV) was found, however, to be inactive in doses up to 10 mg.[14] Thus, the other possible speculation was raised: the removal of the C-19 angular methyl group could have played a key role.[3] This initial observation of very high progesterone-like activity for a 19-nor steroid was of great significance since it stimulated further research interest in this area.

A breakthrough in the synthesis of 19-nor steroids came in 1949 when Birch and Mukherji[15] reported that the 3-glyceryl ether of estradiol could be converted to 19-nortestosterone by means of sodium or potassium in liquid ammonia via the 1, 4-dihydro derivative, followed by hydrolysis with mineral acid. A major improvement in the Birch reduction was discovered by Wilds and Nelson[16] who used the 3-methyl ether of estradiol instead of the 3-glyceryl ether, and who used lithium in place of sodium or potassium in liquid ammonia for this reduction (Figure 2). The Birch reduction offered therefore ready access to many 19-nor steroids.

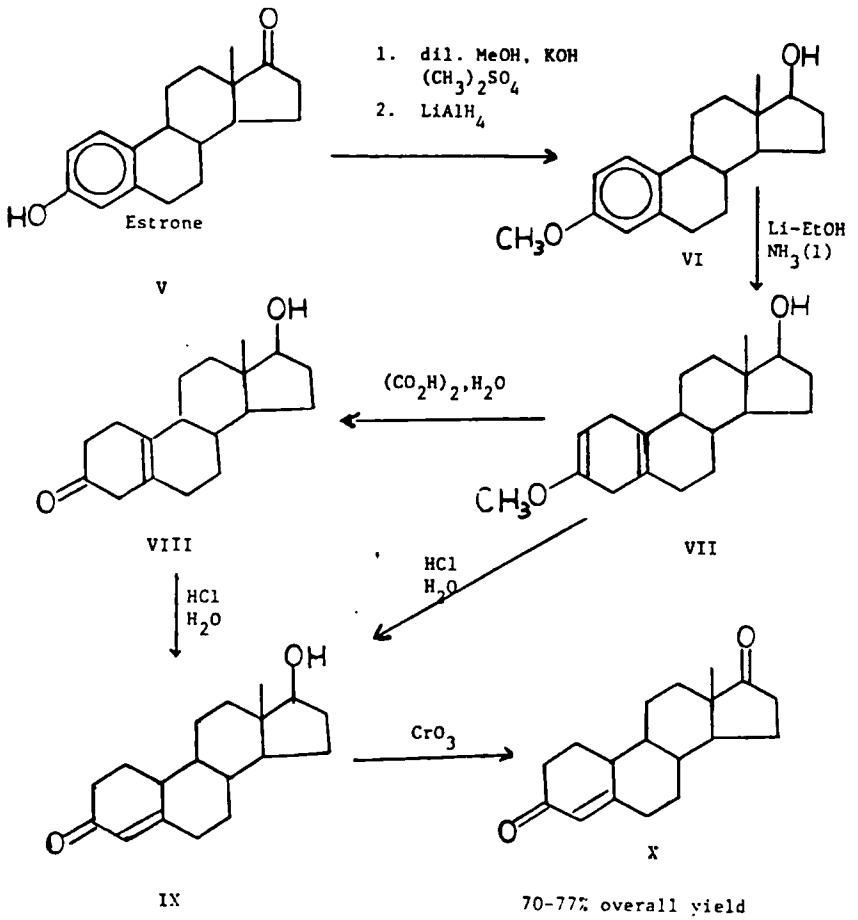

FIGURE 2 Modified Wilds-Nelson Birch reduction.

Djerassi and his coworkers,[17,18] working in the laboratories of Syntex S.A. in Mexico, first applied this improved Birch procedure to prepare crystalline 19-norprogesterone (XIII) of natural configuration from 17β-acetyl-3-methoxyestra-1, 3, 5 (10)-triene (XI), which differed from the natural hormone (I) only in the replacement of the angular methyl group by hydrogen, at C-10 (Figure 3). This substance was found by injection to possess a progestational activity four to eight times greater than that of progesterone in the standard Clauberg rabbit assay for endometrial proliferation.[19] Thus, it became the most potent progestational agent known at that time.

Even though 19-norprogesterone showed considerably greater progestational activity than natural progesterone, 19-nortestosterone, by contrast, was less androgenic than its natural homologue testosterone.[20]

In 1937, 17α-ethynyltestosterone (XVI, ethisterone) was prepared by the addition of potassium acetylide,[21,22] followed by an Oppenauer oxidation using aluminum isopropoxide (Figure 4).[23]

Ethisterone displayed some oral progestational activity in spite of its significant androgenicity. On this basis, it was introduced to the

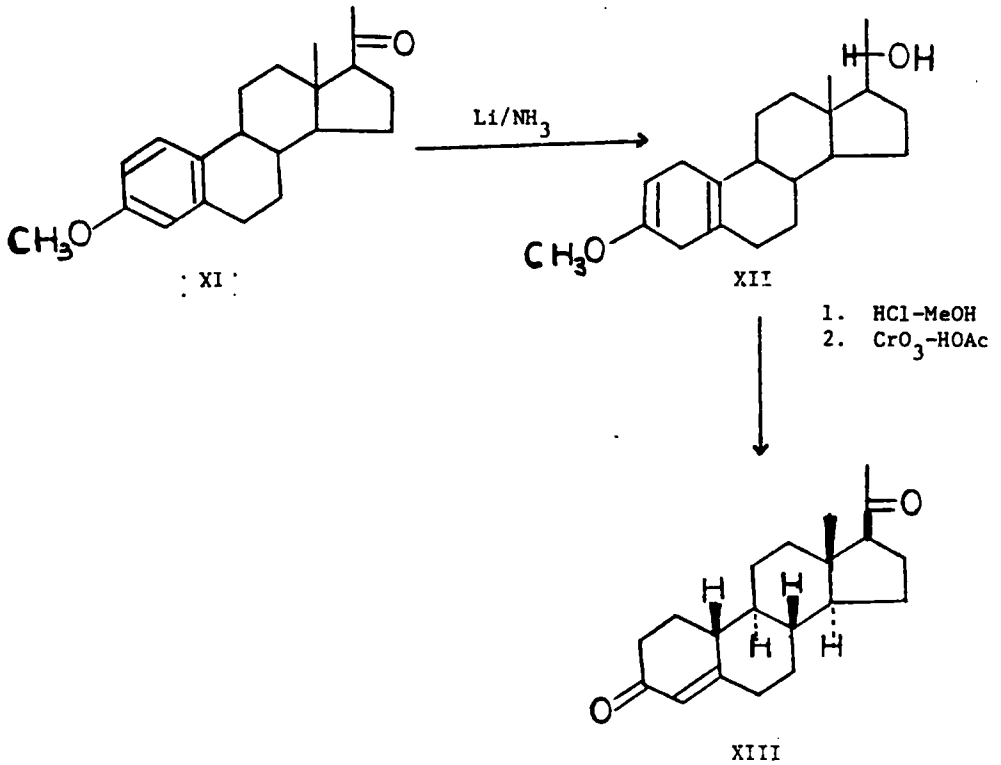

FIGURE 3 Synthesis of 19-norprogesterone.

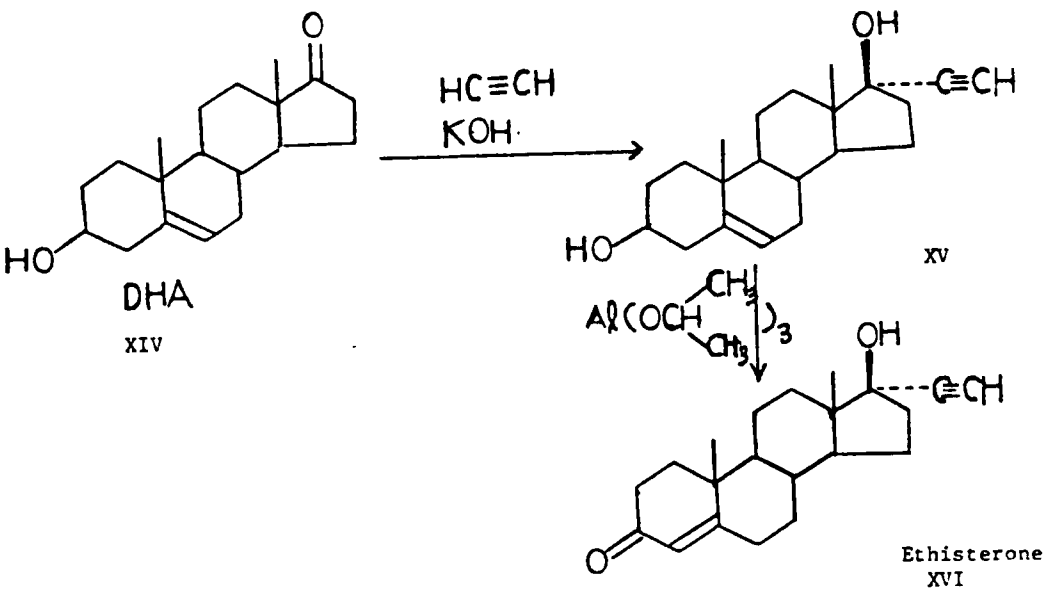

FIGURE 4 Synthesis of ethisterone.

medical profession in 1941 for the treatment of certain menstrual disorders.[6] The oral activity of ethisterone (XVI), coupled with the potent activity of 19-norprogesterone (XIII), prompted investigators to incorporate both modifications into the same molecule. To that end, two very important substances, norethindrone and norethynodrel, were prepared independently by two different laboratories. Norethynodrel was prepared by F. B. Colton of G.D. Searle,[24] while norethindrone was synthesized by C. Djerassi of Syntex Laboratories.[25] The credit for demonstrating the effectiveness of the estrogen-progestin oral contraceptive must unquestionably be given to the late Dr. Pincus and his colleagues. Their initial studies, which established the antiovulatory properties of the 19-norprogestins in animals[26] and human females,[27] were reported in 1956.

Early in 1953, Pincus, Chang, and collaborators at the Worcester Foundation for Experimental Biology began to evaluate norethindrone, norethynodrel, and related analogues for antiovulatory activity. They established that activity for the first time.[26] Subsequently, Rock in Boston and Garcia in Puerto Rico successfully applied these new drugs in the clinic.[27] Most important was the discovery that norethindrone showed oral progestational activity five times greater than that of progesterone in rabbits; this result was confirmed by other investigators in rabbits, monkeys, and women.[28,29] A large-scale clinical evaluation of both norethynodrel and norethindrone showed that the oral administration of these progestins, in combination with 17α-ethynylestradiol (EE) or its methyl ether, mestranol (MEE), from the fifth day of the menstrual cycle for 20 or 21 days, prevented pregnancy with almost 100 percent effectiveness.[29] In the early studies, it was thought that the progestins alone were effective. It was soon realized, however, that part of the effectiveness resulted from an estrogen contaminant, MEE, a precursor in the synthesis of the 19-norprogestins.[30] The superiority of the estrogen-containing preparations over the nonestrogenic progestin was soon revealed in controlling breakthrough bleeding. This vital clue led to the development of currently available estrogen-progestin combination products.[27,31] Not only did the added estrogen permit smaller doses of the more expensive progestin, but it counteracted any potential antiestrogenic effect of the progestin and sustained better integrity of the endometrium.

Large-scale clinical trials soon established the efficacy and safety of these combinations. The era of oral contraception began in 1960 when the combination product Enovid was approved by the U.S. Food and Drug Administration (FDA) for the cyclic control of ovulation and was immediately marketed for this use by Searle.[6] In 1962, the FDA extended its approval to the combination of norethindrone and mestranol for the control of ovulation. This substance was introduced to the medical profession in 1963 by Ortho Pharmaceutical Corporation under a license from Syntex.[6]

PARTIAL SYNTHESIS OF 19-NOR STEROID DERIVATIVES

As shown in Figure 5, most steroid drugs are, in fact, prepared by partial synthesis from natural products that contain the steroid nucleus. The bulk of the world's supply of steroid starting material is derived from only two species of plants: Mexican yam, a species of Dioscorea, and soybean.

Degradation of the Side Chain at C-17

In a process developed by the chemists at Syntex Laboratories, the sapogenin diosgenin was obtained from the Mexican yam.[32-34] This material, with the requisite tetracyclic nucleus possessing the correct stereochemistry, contains eight carbon atoms in the side chain at C-17.

Treatment of diosgenin with hot acetic anhydride leads to a reaction similar to the formation of enol ethers from ketals. In this case, the net result of the transformation is the opening of the spiran

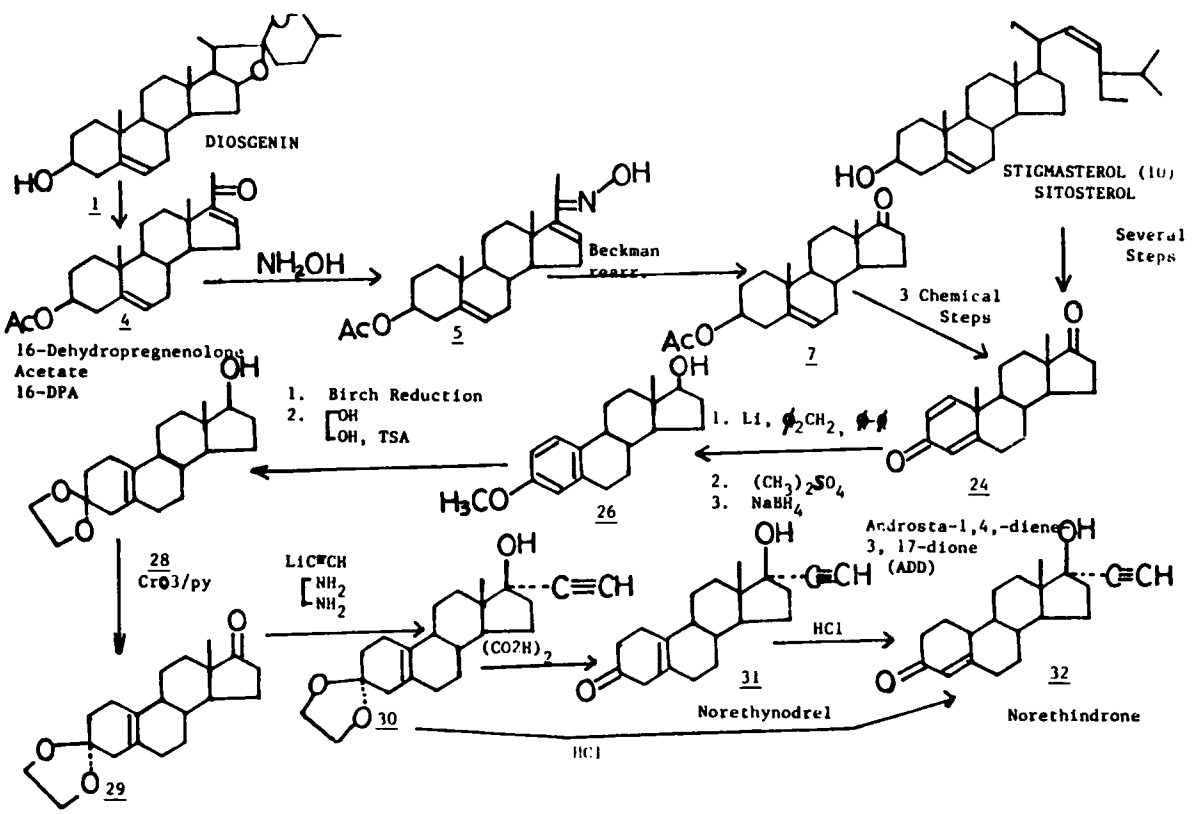

FIGURE 5 Partial synthesis of 19-nor steroids.

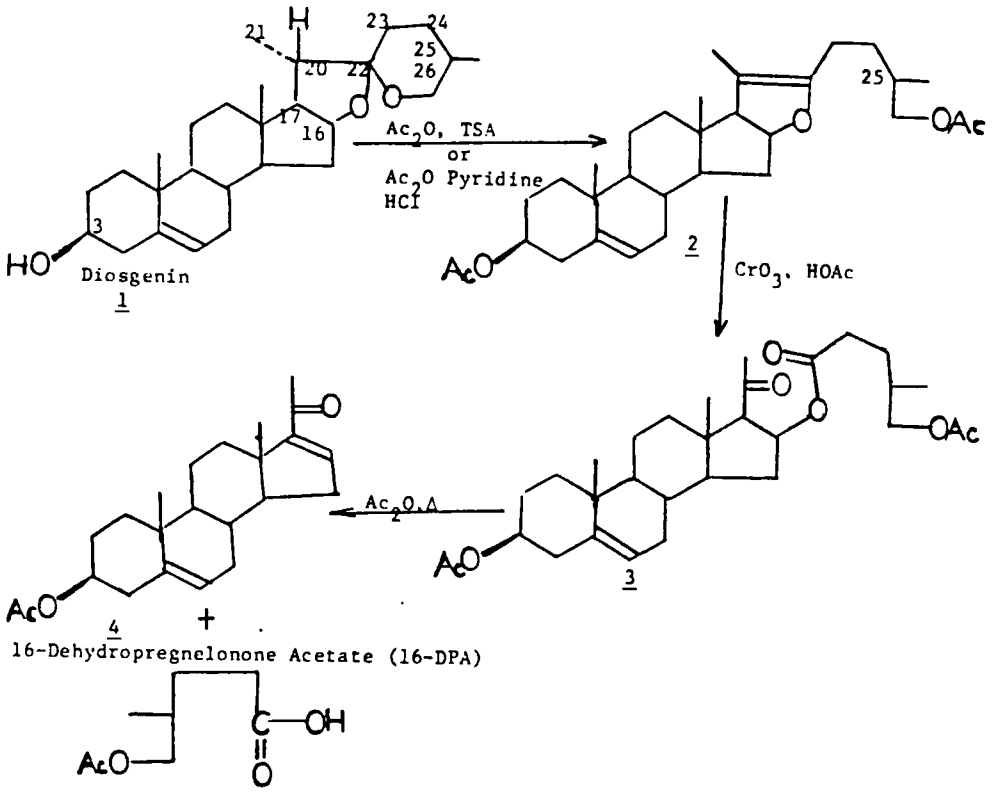

FIGURE 6 Degradation of the side chain of sterols.

ring to a dihydrofuran ring; the hydroxyl group at C-26 is acylated under these conditions (Figure 6).[33,34] Oxidation of 2 with chromium trioxide in acetic acid effects the desired scission of the side chain with the formation of the desired C-20 ketone (3). Treatment of this ester, 16-acyloxy-20-keto steroid (3), with acetic anhydride leads to the elimination of the 16β-γ-acetoxymethylvarleroyloxy grouping. Thus obtained is an intermediate with functionality suitable for subsequent modification, 16-dehydropregnenolone acetate (4). As shown in Figure 7, 4 is converted to its oxime; treatment of this anti-20-oxime (5) under the conditions of the Beckmann rearrangement results in migration of the unsaturated center to nitrogen with consequent formation of the acylated enamine (6).[35] This reaction must involve migration of the vinylic C-17 carbon in anti-configuration of the oxime. Therefore, this rearrangement conforms to the accepted geometrical requirement for migration of the group trans to the departing anion. (On the other hand, a syn-20-oxime would suffer C-21 methyl migration to give the N-methylamide.) No explanation is apparent here. The N-acetylenamine (6) is very unstable with water and readily hydrolyzes to give dehydroepiandrosterone (DHA) acetate (7). Removal of the 3-acetate by saponification followed by Oppenauer oxidation affords the conjugated enone (9). Thus, androstenedione (9) is obtained.[36] This is one of the best-known processes for degrading a pregnane into an androstane derivative on a production scale.

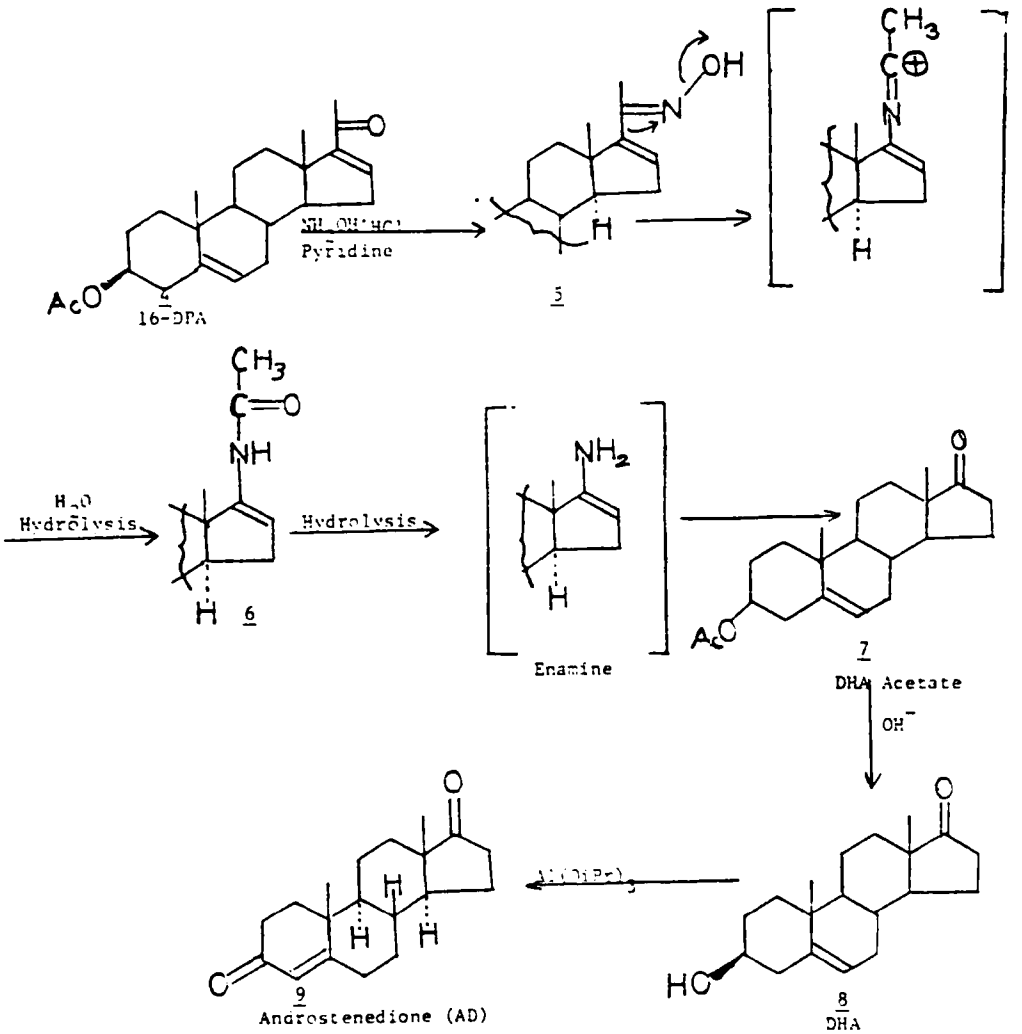

FIGURE 7 Conversion of 16-dehydropregnenolone to androstenedione.

The nonsaponifiable fraction from the abundant oil of soybeans, one of the principal agricultural crops of the United States, is known to be rich in a mixture of sterols bearing a 10-carbon side chain at the 17 position. The chemists at Upjohn Company selected stigmasterol, which is susceptible to chemical degradation given the presence of unsaturation in the side chain,[37,38] and they used it to prepare progesterone,[37] which becomes a starting material for the production of medroxyprogesterone acetate[39] for maintenance of pregnancy. A new process for the isolation of stigmasterol of about 88 percent or better purity from soybean sterols via the discontinuous countercurrent leaching process was described by Campbell and coworkers in 1957.[40] Use of a by-product is an important consideration in the selection of a raw material for steroid production, since a by-product almost always gives superior economics. Soybean serols are about 20 percent stigmasterol.

Stigmasterol (10) is converted into progesterone (I) by using an efficient four-step process that involves: (1) Oppenauer oxidation of 10, (2) selective and stereospecific ozonolysis of the most electron-rich double bond in the side chain in the presence of pyridine,[38] (3) 20-enol acetate formation, and (4) ozonolysis followed by hydrolysis of any C-3 enol ester (see Figure 8).

The other sterols with the saturated side chain at C-17, mostly campesterol and sitosterol, were of less interest until recently. Now, however, microbial methods for the degradation of sitosterol and campesterol to useful steroid intermediates are being developed.

Many methods for the conversion of pregnanes to androstanes are available. The simplest, most practical, and most widely used method on a production scale is the Beckmann rearrangement of Δ^{16}-20-oximinopregnenes (Figure 7).

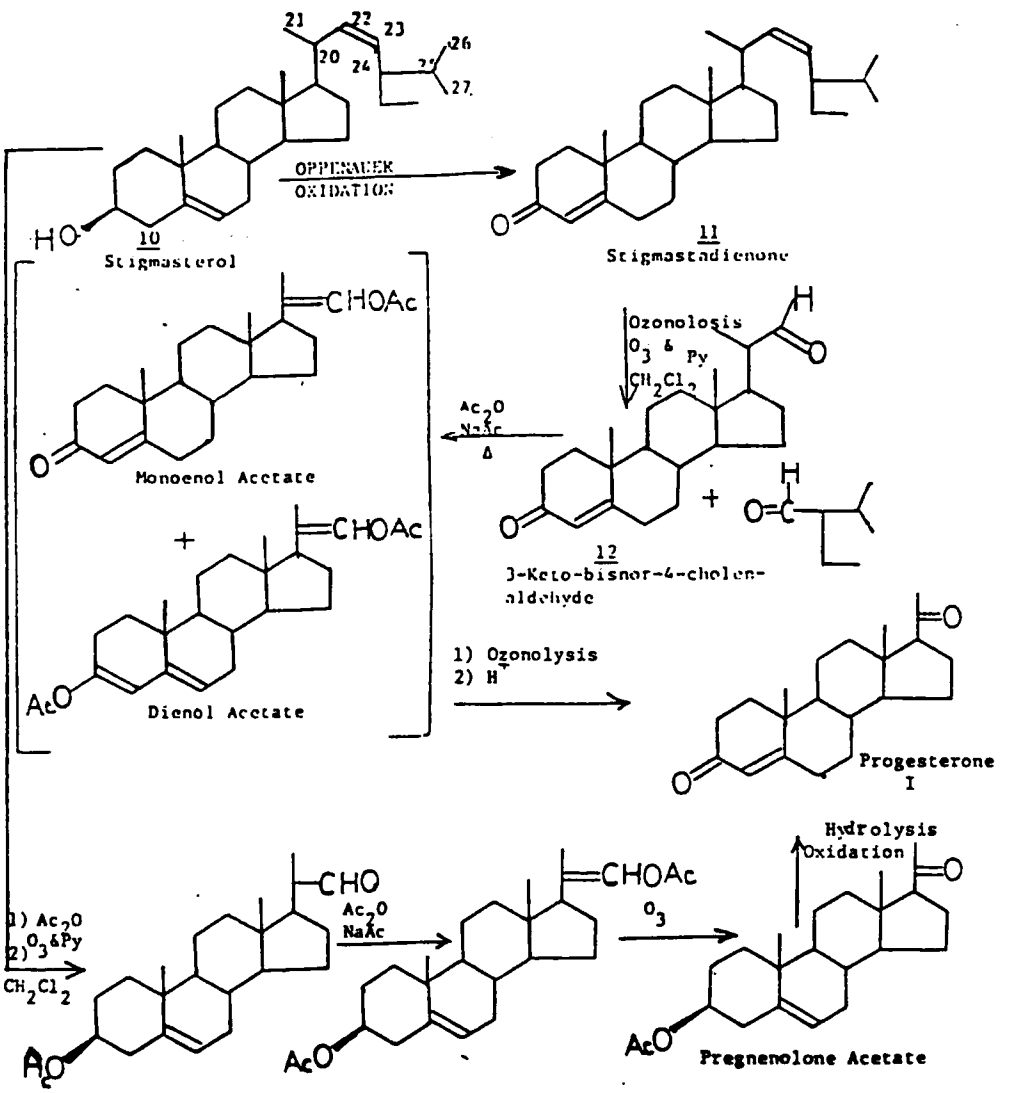

FIGURE 8 Degradation of stigmasterol to progesterone.

Ketalization of 16-DPA (4), one of the early intermediates from the diosgenin route (Figure 6) to form $\Delta^{5,16}$-pregnadiene-3β -ol, 20-one ethylene ketal was followed by saponification to give the alcohol (13). An Oppenauer oxidation on this product followed by epoxidation with perbenzoic acid[41] (or commercially available peracetic acid[42,43]) yielded Δ^{4}-pregnene-3, 20-dione-16α, 17α-epoxide 20-ethylene ketal (15). Condensation of this mono-ketal with ethylene glycol afforded Δ^{5}-pregnene-3, 20-dione-16 , 17 -epoxide bisethylene ketal (16), which upon treatment with $LiAlH_4$ followed by HCl was converted directly to 17α-hydroxyprogesterone (17) (Figure 9).

One of the most widely used methods for the introduction of a 17α-hydroxy group into a 20-ketopregnane involves the protection of a 3-keto-Δ^{4} system via its eniminium salt, through a dienamine, permitting enol acetylation of a 20-ketone, epoxidation, hydrolysis, and finally removal of the A-ring protecting group.[44] Since many steroid double bonds will react with peracids, they must be protected before the enol acetylation step. It is expected that a mixture of the cis (Z) and trans (E) $\Delta^{17(20)}$-enol acetates is obtained. No substantial difference in reactivity of the two isomers is shown here, however. Furthermore, the configuration of the acetyl side chain (α or β) in the starting material is of no consequence, since the asymmetry at C-17 is destroyed by double bond formation. Epoxidation

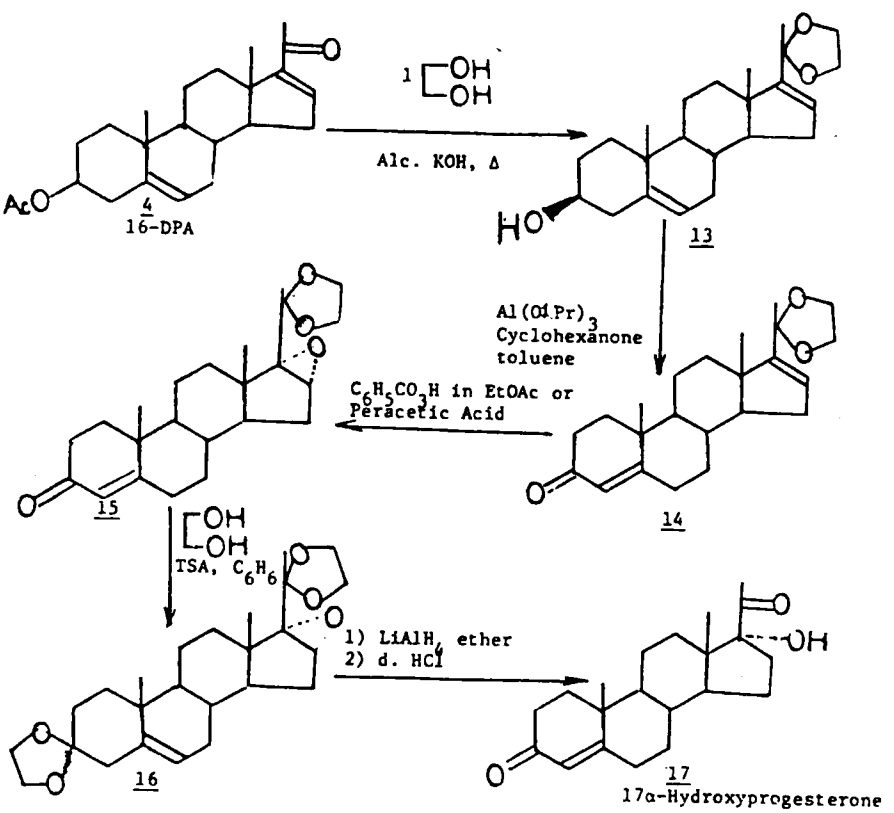

FIGURE 9 Conversion of 16-DPA to 17α-hydroxyprogesterone.

of the $\Delta^{17(20)}$ enol acetate was originally carried out with perbenzoic acid,[41] but the commercially available peracetic acid is generally the most convenient.[42,43] Based on the expected backside attack, the desired epoxides have the 17α-configuration, and hydrolysis always produces the 17α-hydroxy group. The opening of the epoxide and regeneration of the 4-ene-3-one can be carried out simultaneously by treatment with 0.2 N aqueous ethanolic sodium hydroxide to give 17-hydroxypregn-4-ene-3, 20-dione (17) in 48 percent overall yield from progesterone (Figure 10). This demonstrates the stability of eniminium salts which may prove valuable when selective protection of an α,β-unsaturated carbonyl group is required during an electrophilic reaction. The protection of Δ^5 double bond is available by halogenation during the introduction of 17α-hydroxyl group prior to enol acetylation.[45]

In 1962, another elegant procedure was devised by Barton[46] for the introduction of 17α-hydroxyl group into a 20-ketopregnane by $tBuO^-/O_2$/Zn-HOAc procedures. As is so often the case, the method arose as a matter of commercial necessity. Subsequently improved, Barton's procedure recommends the use of t-butoxide in DMF-t-butanol at -20°C to -25°C in the presence of triethyl phosphite to reduce the hydroperoxide formed. Although a 3-keto-Δ^4 system is not stable to the reaction conditions, a 3-keto-$\Delta^{1,4}$ system will survive.[47] Treatment of progesterone with DDQ in dioxane affords 21 (see Figure 11).[48]

Edwards and coworkers at Syntex Laboratories described the cleavage of 17α-hydroperoxypregnenolone with potassium t-butoxide in THF to give DHA in good yield.[49] Since such hydroperoxides are now accessible

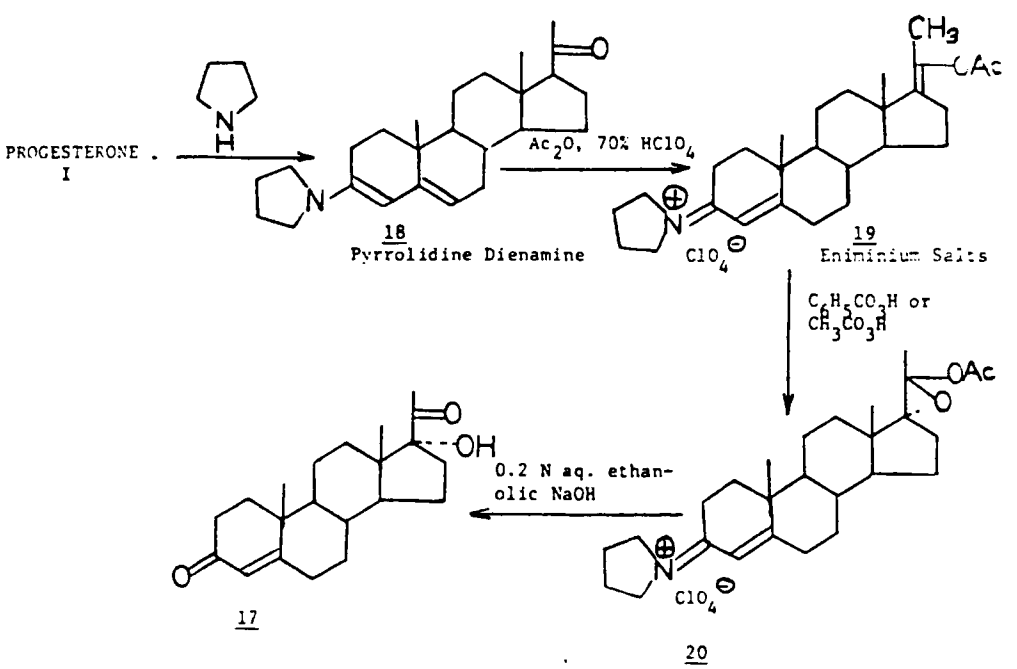

FIGURE 10 Introduction of a 17-hydroxy group into progesterone.

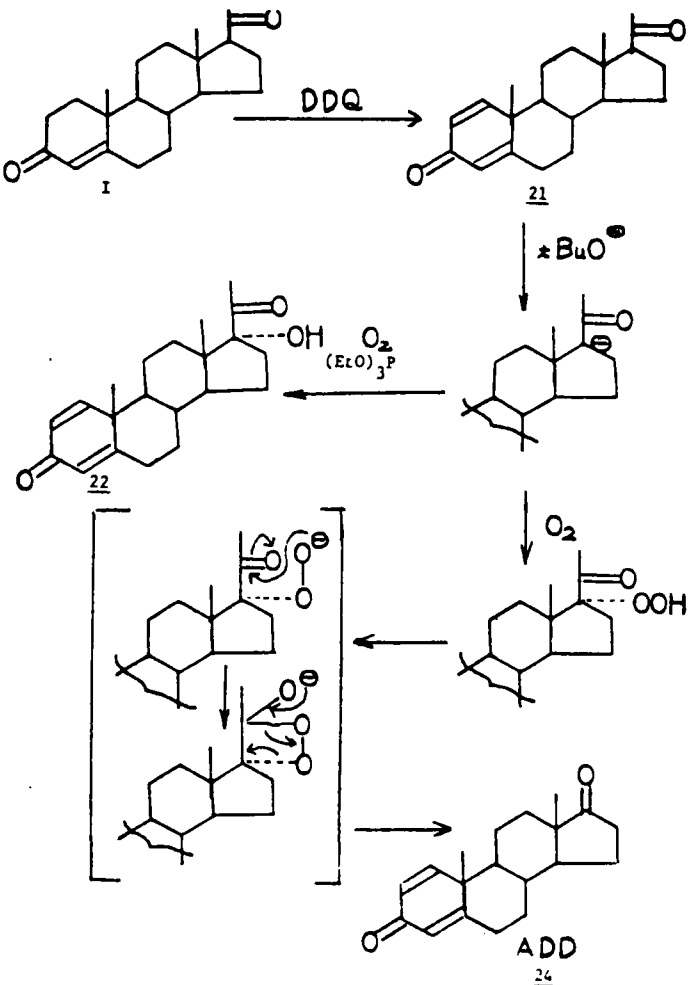

FIGURE 11 Direct oxygenation of 20-ketopregnanes.

from the 20-ketopregnanes in one step, this constitutes a convenient two-step degradation process. In practice, the intermediate hydroperoxides need not be isolated. Other enolizable keto groups in the molecule must be protected (see Figure 12).

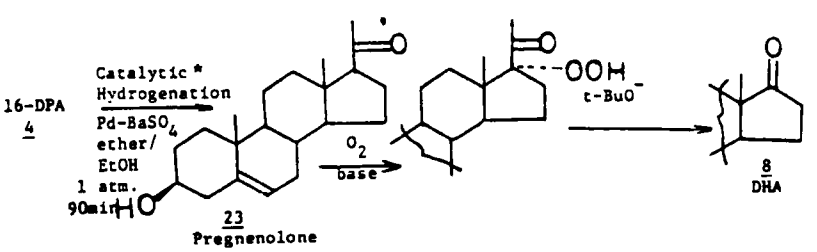

FIGURE 12 Degradation via 17-hydroperoxides (*R. E. Marker and J. Kruger. 1940. Sterols. CXII. Sapogenins. XLI. The preparation of Trillin and its conversion to progesterone.J. Am. Chem. Soc. 62:3349.).

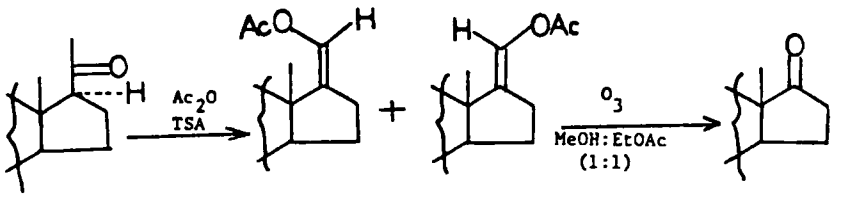

FIGURE 13 17-ketosteroids from enol acetates of 20-ketosteroids.

Similar 17-ketosteroids are obtained by means of ozonolysis on two stereoisomeric enol acetates, cis (Z) and trans (E), about the double bond of 20-ketosteroids (see Figure 13).[50]

17α-hydroxypregnenolone 3-acetate can be cleaved quantitatively to DHA acetate (7) using lead tetraacetate ($Pb(OAc)_4$) in an aprotic solvent such as benzene at reflux (see Figure 14).[51]

In addition, a variety of oxidative reagents, including chromium trioxide, lead tetraacetate, periodic acid, and sodium bismuthate, can be used to cleave the 17,20-oxygenated pregnanes.[52]

Aromatic A-Ring Steroids

Treatment of androstenedione (9) with DDQ[48] leads to the formation of 1,4-dienone (24). Aromatization is accomplished by treatment of the 17-ethylene ketal of 1, 4-dienone (24) with an excess of the radical anion derived from lithium metal and biphenyl in boiling THF solution. Since the angular methyl group in this case leaves as methyl lithium, diphenylmethane is included in the reaction mixture to

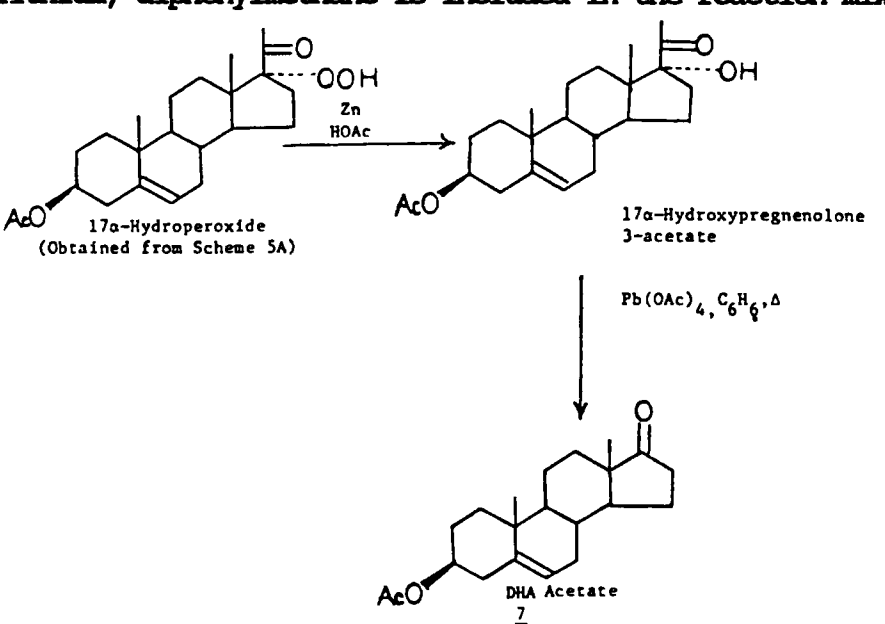

FIGURE 14 The degradation of C-21 to C-19 steroids.

quench this by-product, thereby preventing its reaction with the starting material.[53] Acidification of the reaction mixture hydrolyzes the ketal function and affords estrone in quite a respectable yield (Figure 15).

19-Nor Steroids

Estrone is converted to its 3-methyl ether by reaction with dimethylsulfate.[16] Reduction with sodium borohydride in THF/EtOH gives the 17β-alcohol.[16] Estradiol 3-methyl ether (26) is the starting material for the synthesis of norethynodrel (31)[24] as well as norethindrone (32).[25]

The Birch reduction of 26 with lithium in liquid ammonia using THF and t-butanol as cosolvents results in the formation of the 1, 4-dihydro derivative (27), which is subsequently interchanged with ethylene glycol in the presence of TSA (p-toluenesulfonic acid) and converted to the more stable ethylene ketal compound (an improved procedure).[54] Oxidation of 28 with the chromium trioxide-pyridine ($CrO_3.2C_5H_5N$)[55] results in a good yield of the 17-ketone (29) without aromatization. Ethynylation of the 17-ketone of 3-ethyleneketal (29) with a metal acetylide, such

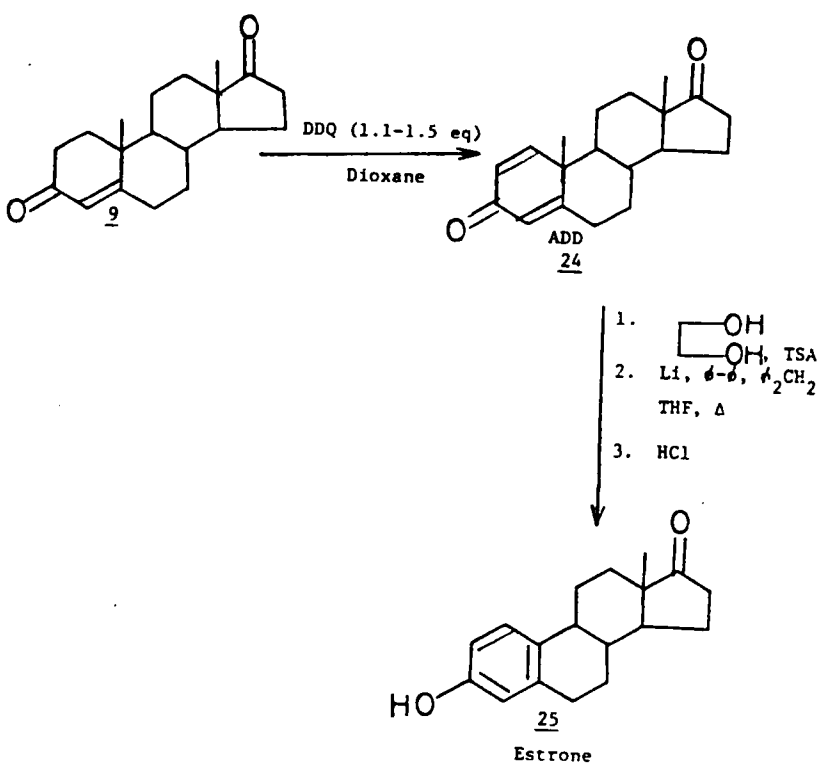

FIGURE 15 Reductive aromatization of dienones.

as commercial lithium acetylide ethylenediamine complex, gives the 3-ethyleneketal of norethynodrel (30). Mild acid hydrolysis (using, for example, the oxalic acid of the latter) produces norethynodrel (31), whereas hydrolysis with hydrochloric acid gives norethindrone (32), as shown in Figure 16.

Functionalization at the C-19 Position

Until about 1961, the synthesis of 19-nor steroids was almost entirely based on the Birch reduction of estrone 3-methyl ether. The commercial importance of steroid oral contraceptives prompted extensive work on alternate partial syntheses which do not proceed via a Birch reduction of ring A aromatic precursors. A very efficient procedure was discovered simultaneously by two groups working independently in Mexico[56,57] and Switzerland,[58,59] who approached the problem through chemical functionalization of the angular methyl group at position 10.

The procedure described by Kalvoda et al.[59] of Ciba AG is representative of these new processes and is especially suitable for the preparation of norethynodrel (31) and norethindrone (32). The

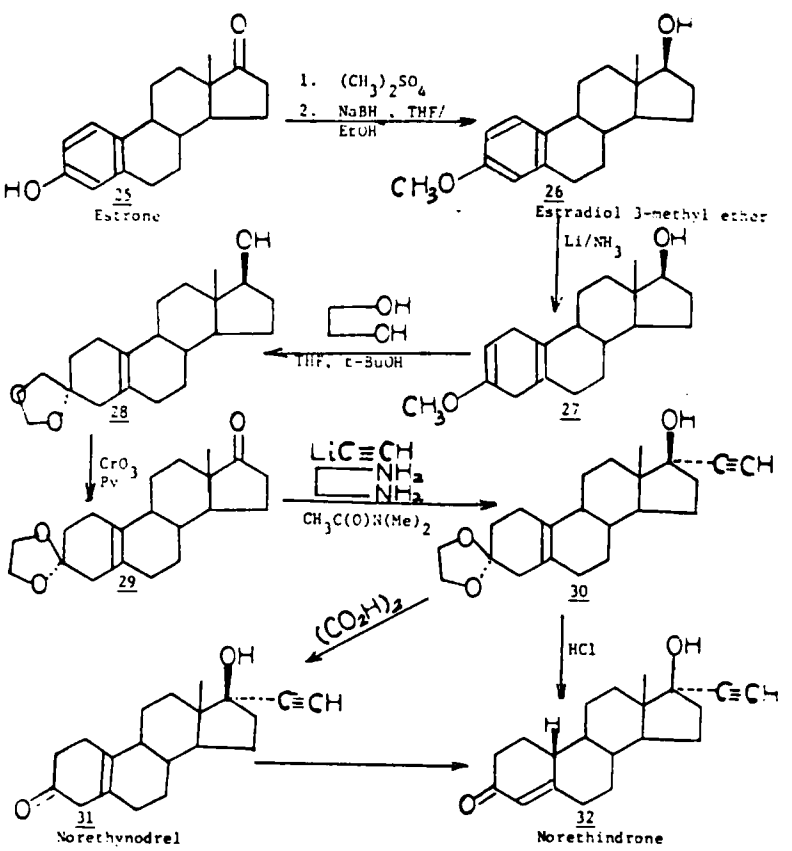

FIGURE 16 Synthesis of 19-nor steroids.

addition of hypochlorous acid to 3β-hydroxyandrost-5-en-17-one (DHA) acetate (7) results in an excellent yield of the chlorohydrin. The C-10 methyl group is then functionalized by treatment with lead tetraacetate to give the 6β, 19-oxide. Hydrolysis of this oxide followed by oxidation gives the dione. Subsequent alkali treatment produces the 4-dehydro-3-keto derivative. Reduction of the 4-dehydro-3-keto compound with zinc affords the important, intermediate 19-hydroxyandrost-4-ene-3, 17-dione (33). Treatment of this dione with chromic acid provides the acid (34) that upon heating in pyridine is decarboxylated to give the 5(10)-dehydro derivative, β,γ-unsaturated ketone (35). Treatment of 35 with a weak acid in methanol results in selective ketalization at C-3 to give the 3-dimethylketal (36). Subsequent ethynylation at C-17 produces an excellent yield of the 3-dimethylketal (37) of norethynodrel. Weak acid cleavage of the dimethylketal (37) yields norethynodrel (31), while a more vigorous acid treatment gives norethindrone (32). Although many steps are involved in this transformation, each of the reactions results in a good yield, and a high overall conversion to the desired product is obtained. This procedure is very attractive for the large-scale preparation of 19-nor steroids. The inconvenient Birch reduction of ring A aromatic precursors for large-scale operations is totally avoided here when compared with Djerassi's original procedure involving a Birch reduction (25). This alternate approach can be used for a small-scale preparation in the laboratory for conversion of DHA (8) to norethindrone (32). Several modifications of the C-19 functionalization of Kalvoda have been reported (see Figure 17).[58]

Bowers et al.[56] at Syntex utilized the bromohydrin instead of chlorohydrin and reported good yields by converting the intermediate, 6β, 19-oxido-5α-bromoandrostane-3, 17-dione to 19-hydroxyandrost-4-ene-3, 17-dione (33) using a zinc and isoprophyl alcohol treatment followed by acid isomerization of the C-5 double bond, in a manner similar to that of Kalvoda's procedure (Figure 17).

Another improvement in this general scheme was worked out by Pappo and Nysted of G. D. Searle (Figure 18).[60] This involved the peracid formation of the 5,6α-epoxide, followed by dilute perchloric acid cleavage to the 5,6-diol. Acetylation gave the 3,5,6-triacetate which reacted selectively with potassium bicarbonate to yield the 3,6β-diol-5α-acetate. Selective acetylation at C-3, and then lead tetraacetate and iodine functionalization of C-19, gave the 6β, 19-oxide. Potassium bicarbonate hydrolysis of the 3-acetate and then chromic acid oxidation of the resulting alcohol gave the key intermediate 6β, 19-oxido-5α-acetoxyandrostane-3, 17-dione. Treatment of 6β, 19-oxido-5α-acetoxyandrostanedione with zinc and zinc chloride in methanol gave a good yield of 19-hydroxyandrostenedione (33). Oxidation with chromium trioxide converted 19-hydroxyandrostenedione (33) into the C-10 carboxyΔ^4-3-keto compound. Upon gentle heating in aqueous pyridine, the acid was decarboxylated to give the 3-keto-$\Delta^{5(10)}$ steroid (35). The overall yield for this reaction sequence was good. The need for metal-ammonia reduction (Birch reduction) is thus avoided.

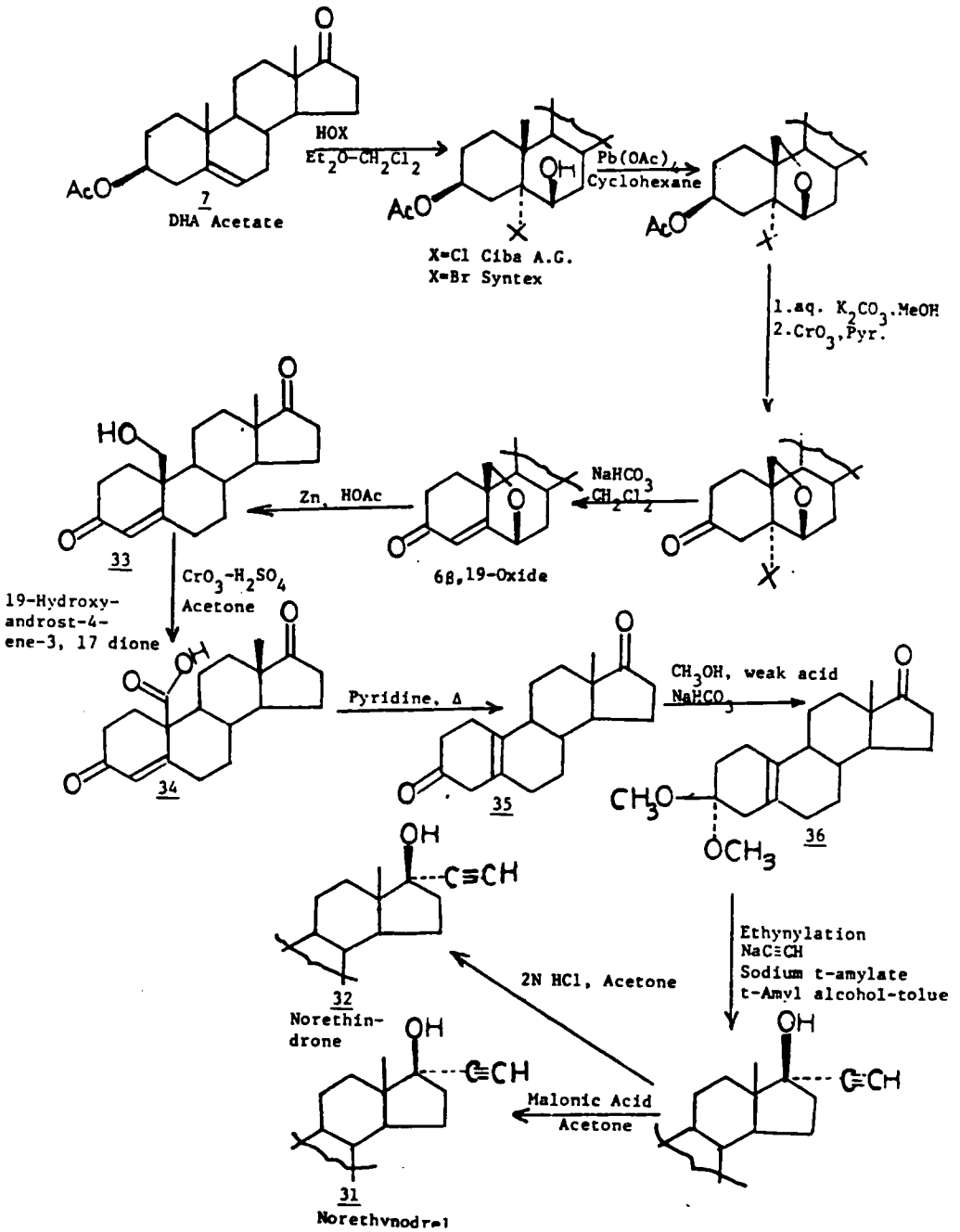

FIGURE 17 Ciba AG, Basel, and Syntex procedures.

Other Modifications of 19-Nor Steroids

Norethindrone Acetate (38)

Esterification of the 17β-hydroxy group of norethindrone provides derivatives with interesting biological properties. The 17-acetate

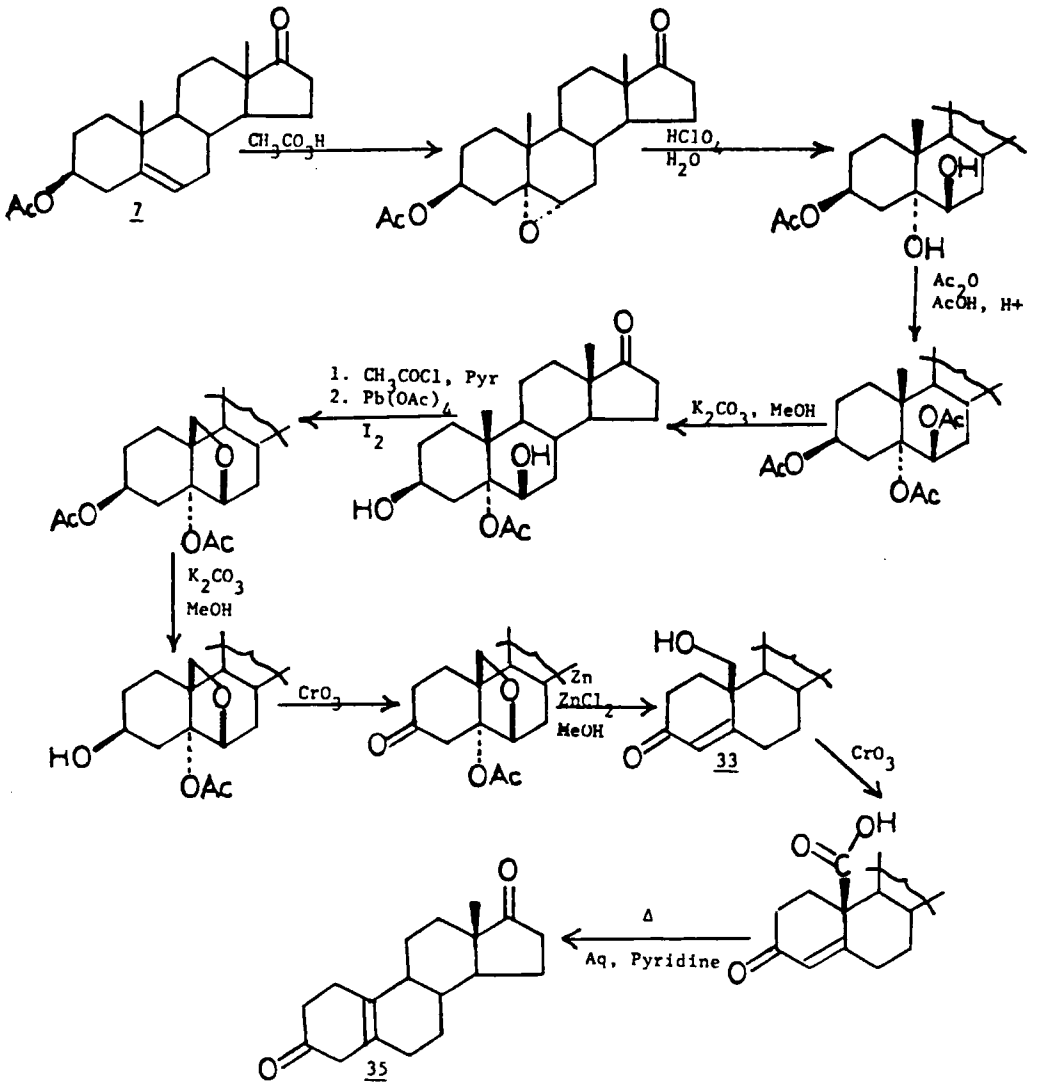

FIGURE 18 G. D. Searle procedure.

ester (38) of norethindrone (32) is orally effective clinically[61] and, with a small amount of estrogen, is marketed in Europe by Schering AG as Anovlar and in the United States by Parke, Davis as Norlestrin (Table 1). One of the synthetic schemes currently used for preparing the 17-acetate involves conversion of norethindrone to the 3, 17-diacetate (33) by treatment with acetic anhydride or isopropenyl acetate in the presence of p-toluenesulfonic acid as a catalyst.[62] Mild alkaline or acid treatment of the enol diacetate (33) produces norethindrone acetate (38) as shown in Figure 19. An alternate method for the preparation of the 17-acetate (38), published in 1968 by Shapiro and coworkers,[63] involves ethynylating and acetylating the enol ether in the same reaction and then hydrolyzing it to give the

TABLE 1 Combined Oral Contraceptives Marketed in the United States (as of October 2, 1986)

Brand Name (Day Regimen)	Company	Progestin	Estrogen
Ortho-Novum 1/50 (21,28)	Ortho	1.0 NET	0.050 MEE
" 1/80 (21,28)	"	1.0 "	0.080 "
" 2 mg (21)	"	2.0 "	0.100 "
Ortho-Novum 7/7/7 (21,28)	"	0.5/0.75/1.0 "	0.035 EE
" 10/11 (21,28)	"	0.5/1.0 "	0.035 "
Ortho-Novum 1/35 (21,28)	"	1.0 "	0.035 "
Modicon (21,28)		0.5 "	0.035 "
Demulen 1/35 (21,28)	Searle	1.0 Ethynodiol Diacetate	0.035 EE
Demulen 1/50 (21,28)	"	1.0 "	0.050 EE
Enovid-E 21	"	2.5 Norethynodrel	0.100 MEE
Enovid 5 mg	"	5.0 "	0.075 "
Enovid 10 mg	"	9.85 "	0.15 "
Ovulen (21,28)	"	1.0 Ethynodiol Diacetate	0.10 "
Brevicon (21,28)	Syntex	0.5 NET	0.035 EE
Norinyl 1+35 (21,28)	"	1.0 "	0.035 "
Norinyl 1+50 (21,28)	"	1.0 "	0.050 MEE
" 1+80 (21,28)	"	1.0 "	0.080 "
" 2 mg	"	2.0 "	0.100 "
Tri-norinyl (21 and 28)		0.5/1.0 "	0.035 EE
Ovral	Wyeth	0.5 dl-Norgestrel	0.05
LO/Ovral (21 and 28)	"	0.3 "	0.030 "
Nordette (21 and 28)	"	0.15 LNG	0.03 "
Triphasil (21 and 28)	"	0.05/0.075/0.125 "	0.03/0.04/0.03 "
Loestrin 1/20	P-D	1.0 NET AC	0.020 "
" 1.5/30	"	1.5 "	0.030 "
Norlestrin 1/50	"	1.0 "	0.050 "
" [Fe] 1/50	"	1.0 "	0.050 "
" 2.5/50	"	2.5	0.050 "

17-acetate (38) (Figure 20). The 3-ethyleneketal (28)--not the enol ether (27)--is preferred during the chromic acid oxidation, as shown in Figure 16.

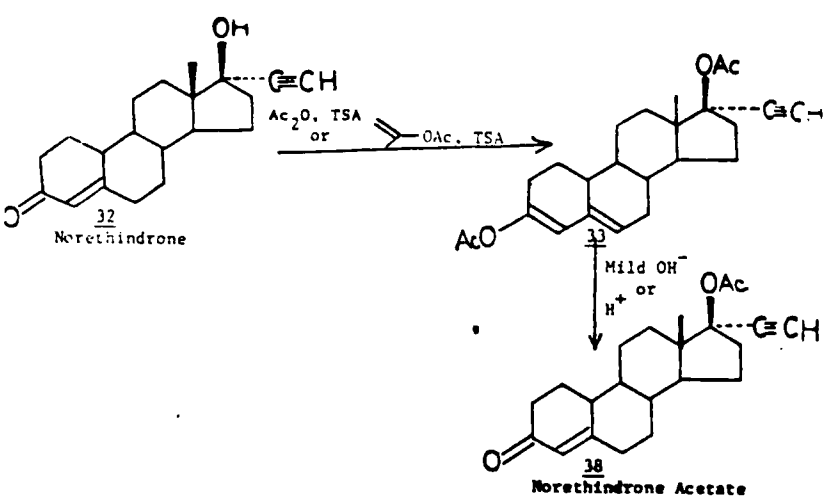

FIGURE 19 Esterification of norethindrone.

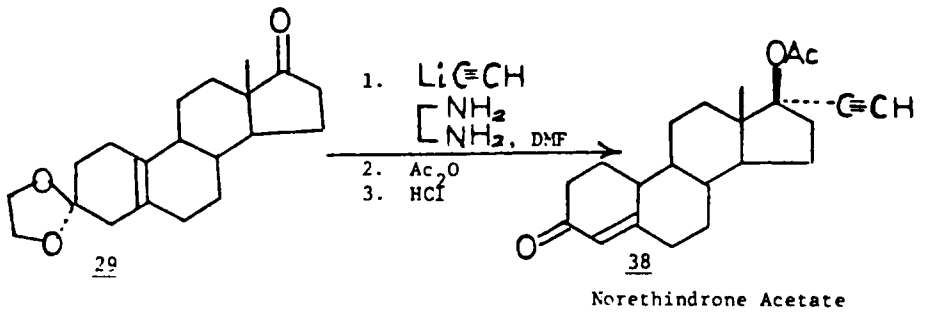

FIGURE 20 Concomitant ethynylation and acetylation.

Since 1975, we have synthesized and tested esters of norethindrone and norgestrel in collaboration with the World Health Organization (WHO).[64] Esterification by using the conventional methods on sterically hindered tertiary alcohols provides a very poor yield of the desired esters. The most successful method appears to be the thallous ethoxide one.[65] The minute amount of toxic thallium residue that remains in the ester is unacceptable and undesirable, however. More recently, a simple mixed anhydride procedure using trifluoroacetic anhydride and the acid for esterification, followed by the reaction of steroid alcohols, was found to be the method of choice for the preparation of steroid esters.

Ethynodiol Diacetate

Good yields of ethynodiol diacetate (40) are obtained by the diacetylation of the diol (39) with acetic anhydride and pyridine.[66] This diol was prepared by Colton[67] and independently by Sondheimer and his coworker[68] by reduction of norethindrone (32) with sodium borohydride. More recently, it was found that lithium tri-t-butoxyaluminum hydride was a superior reducing agent[69] to sodium borohydride or lithium aluminum hydride in producing a greater ratio of the desired 3α-hydroxy isomer over the 3α-hydroxy epimer (see Figure 21).[70]

SYNTHESIS OF ETHYNYLESTRADIOL (41) AND MESTRANOL (42)

Reaction of estrone (25) with a metal acetylide produces 17α-ethynyl-17β-hydroxyestradiol = ethynylestradiol, (EE) (41).[71] This compound is equipotent with estradiol by subcutaneous administration, but it is 15-20 times as active by oral administration. Ethynylation of the methyl ether (25a) of estrone similarly produces mestranol (42) as shown in Figure 22.[72,73]

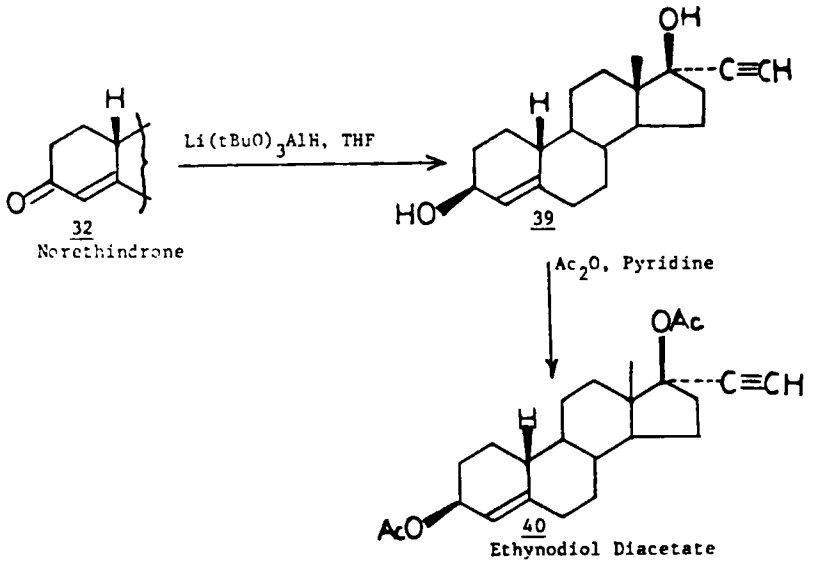

FIGURE 21 Diacetylation of the 3, 17-diol (39).

Attack of the metal acetylide on the 17-ketone takes place from the least hindered α-side of the estrone or estrone methyl ether.

Ethynylestradiol and mestranol are of special commercial significance because most of the oral contraceptives now on sale incorporate one or the other of the compounds as the estrogenic component.

13β-ETHYLGON-4-EN-3-ONES AND RELATED COMPOUNDS BY TOTAL SYNTHESIS

Total synthesis produces not only norethindrone, but also steroids with structures that do not occur in nature. For example, D-(-)-norgestrel, which possesses a 13-ethyl group, is manufactured only by this route. It is probable that intensive process research development on the reactions involved in these syntheses may have made these routes commercially competitive with partial synthesis based on plant sterols.[74]

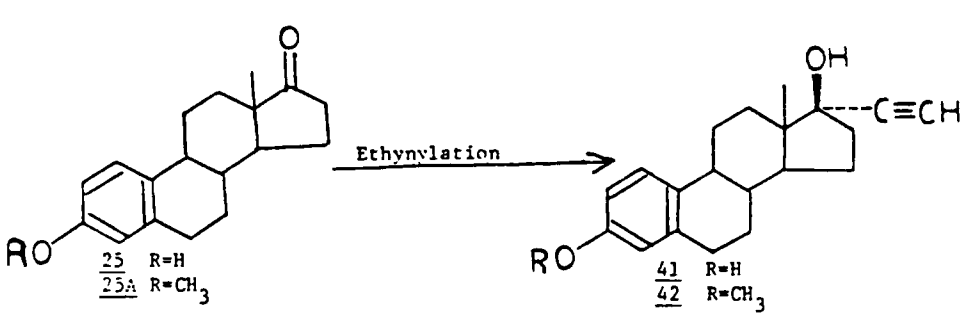

FIGURE 22 Ethynylation of estrone and its methyl ether.

Several useful alternate approaches for the commercial total synthesis of many steroid compounds have been described by Torgov and associates[75-77] and by Smith and coworkers.[79-82] These methods have been utilized to prepare a variety of 13-alkylgona-1,3,5(10)-triene intermediates (Figure 23).

Originally, condensation of 2-ethylcyclopentane-1,3-dione (D) with the vinyl carbincl (AB) obtained from 6-methoxy-1-tetralone and vinylmagnesium chloride, in the presence of about 10 percent of the quaternary ammonium hydroxide Triton B, gave the tricyclic diketone ABD which was ring closed under acid catalysis to ABCD. Catalytic reduction of the 14-double bond proceeds at the α-side because of the presence of the bulky angular ethyl group at C-13. This step has the additional consequence of establishing the important trans C/D ring juncture. Reduction of the 17-ketone proceeds as expected to give the 17β-alcohol. The remaining 8,9-double bond is subjected to further reduction with lithium in liquid ammonia to establish the trans B/C ring juncture. Birch reduction, Oppenauer oxidation of 1,4-dihydroanisole-17β-alcohol, ethynylation, and hydrolysis provide racemic norgestrel.[82] Although this synthesis has involved the

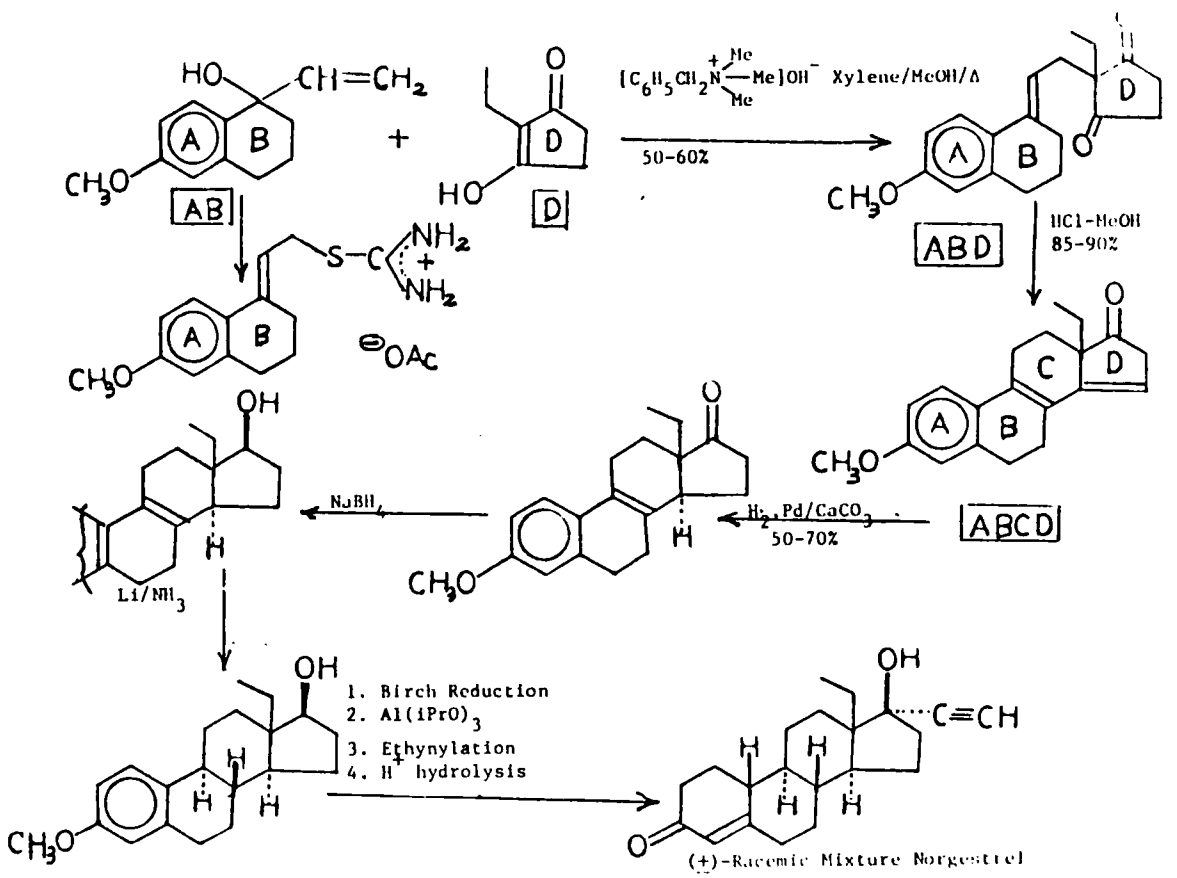

FIGURE 23 Torgov-Smith total synthesis.

formation of six chiral centers, only two of the 64 possible isomers are formed. A major yield improvement resulted from prior conversion of the unstable vinyl carbinol (AB) to the crystalline isothiuronium acetate, which was coupled with an enolate (D) under mild conditions (alcohol) to give the tricyclic diketone (ABD) in 90 percent yield.[83,84]

Total Synthesis of D-(-)-Norgestrel Using a Chirality Inducing Reaction: Schering AG Process

The tricyclic diketone (46) is a prochiral molecule, since reaction at one of the carbonyl groups would create an asymmetric center at C-13 (steroid numbering) as shown in Figure 24. For example,

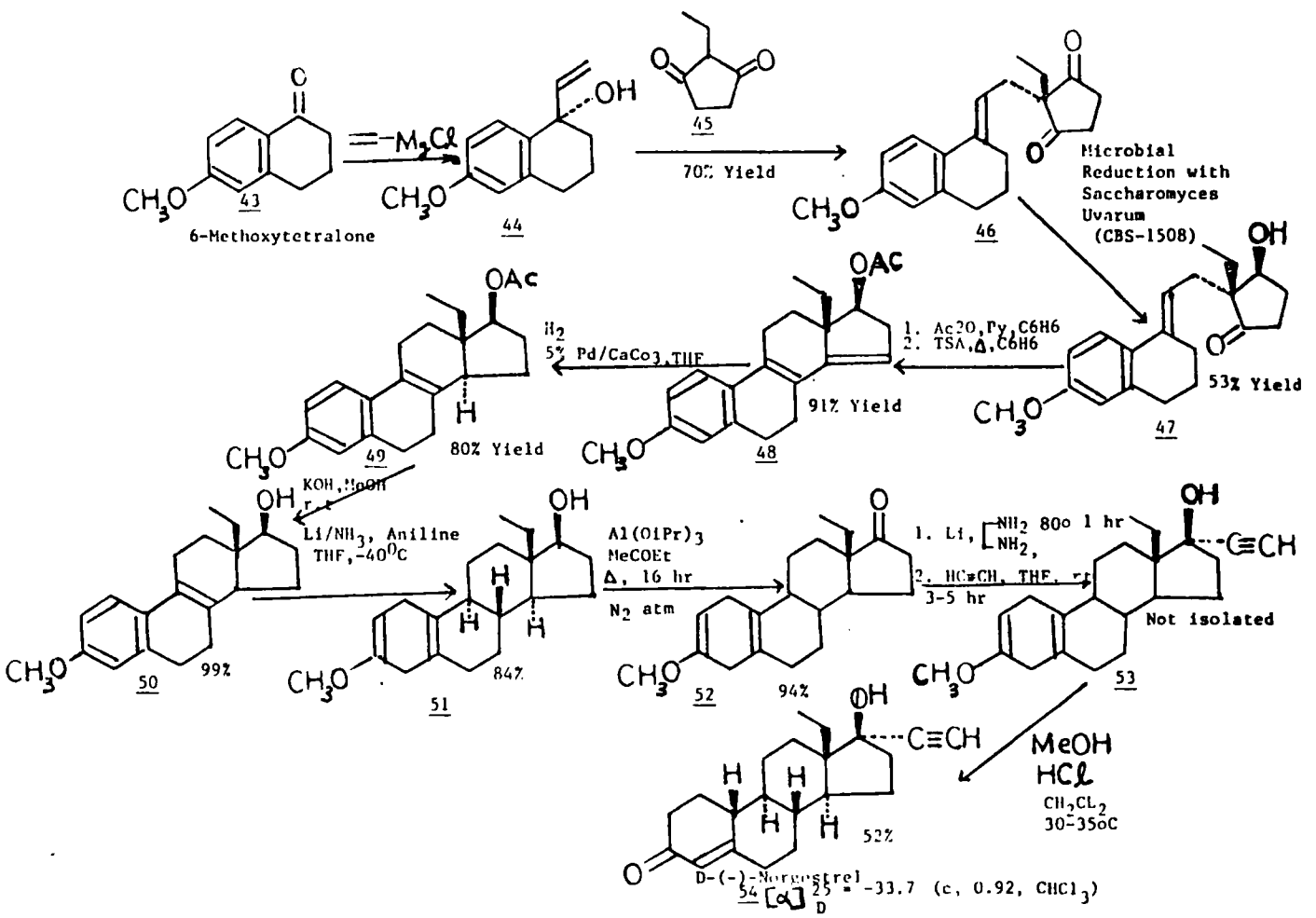

FIGURE 24 Total synthesis of D-(-)-norgestrel using a chirality induction (Schering AG process).

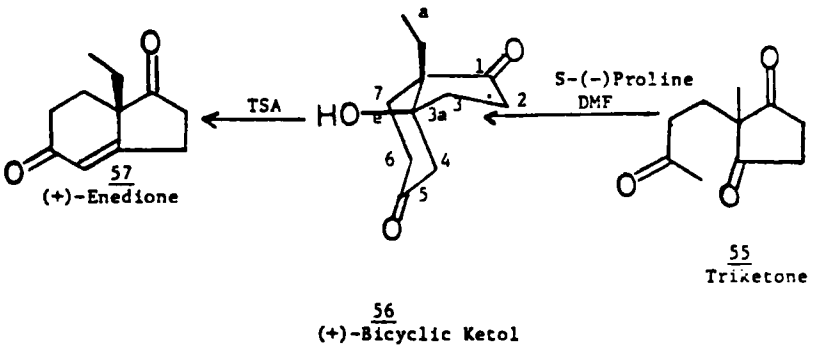

FIGURE 25 Asymmetric aldol cyclization.

microbiological reduction of this tricyclic diketone (46) with the yeast Saccharomyces uvarum (CDB 1508) forms the optically active (-) ketol 47 at a 53 percent yield. The following steps will yield D-(-)-norgestrel: conversion of the 17-alcohol (47) to the 17-acetate, acidic cyclization (48), catalytic reduction of 14,(15)-double bond for the formation of 14α-isomer, hydrolysis of the 17-acetate (49) to the 17-alcohol (50), Li/liquid ammonia reduction of 8,9-double bond, Birch reduction of aromatic ring A portion, Oppenauer oxidation, and ethynylation followed by hydrolysis with methanolic hydrochloric acid. This is the Schering AG process.[85,86] In 1975, a research group at Schering AG published an elegant synthesis as an alternative to this process. It does not proceed via a Birch reduction of the ring A aromatic precursor.

The regioselective sufonylmethylation[87,88] of optically active 7β-ethyl-6H-7, 7a-dihydroindan-1, 5-dione (57) opens the possibility of a new, technically simple synthesis of D-(-)-norgestrel (54).[89]

The (+)-enedione (57) is prepared by dehydration of the (+)-bicyclic ketol (56) in TSA, which is in turn obtained by cyclization of the triketone (55) with S-(-)-proline in DMF as a polar aprotic solvent (Figure 25).[87] Reaction of the (+)-enedione (57) with paraformaldehyde and benzenesulfinic acid in a mixture of triethanolamine[90] and acetic acid in a volume ratio of 3:1[91] at 50°C affords the sulfone (58) at a 85 percent yield. Presence of a larger amount of acetic acid accelerates the reaction, but the regioselectivity is thereby reduced, and considerable amounts of doubly alkylated products of varying structure are formed. In acid solution (ethanol with 1 percent aqueous hydrochloric acid) with palladium on charcoal (10 percent) as a catalyst, the sulfone 58 is hydrogenated to the trans-fused sulfone (59) as a crystalline product in a 75 percent yield.

It is certain that the phenylsulfonyl group is alpha--that is, in an equatorial position. In nonpolar solvents (pentane/benzene),

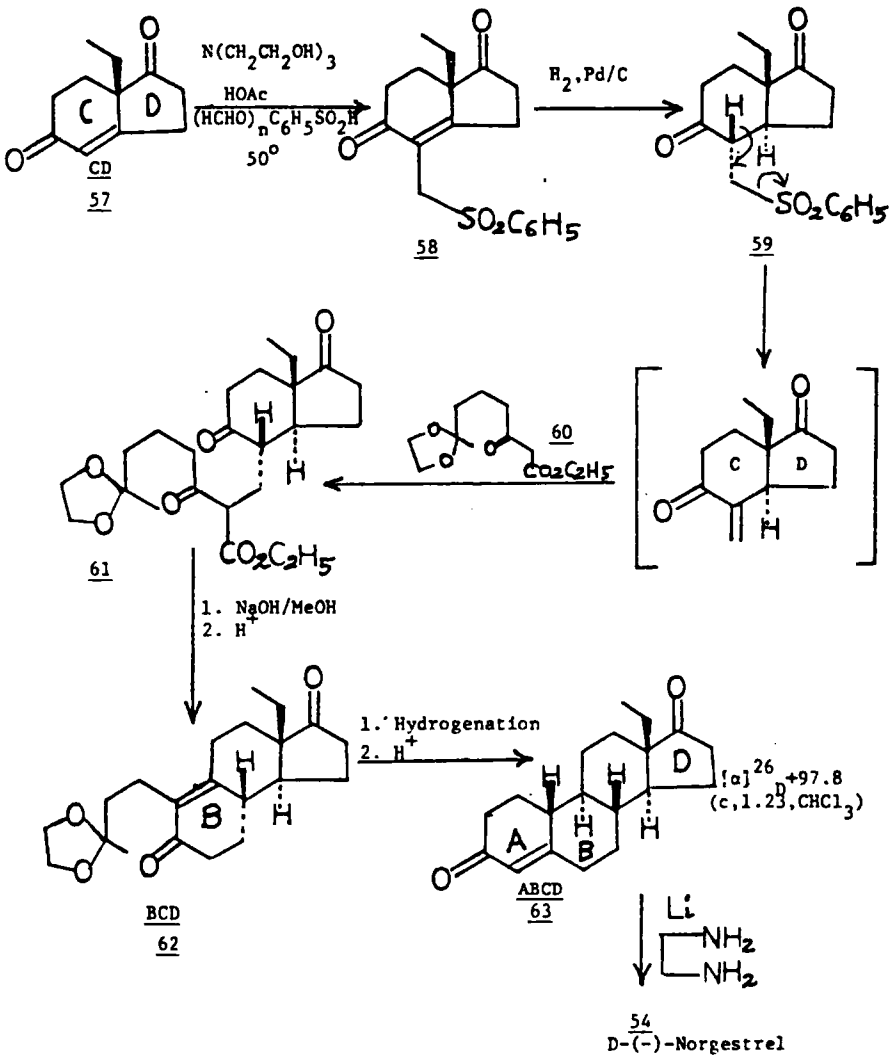

FIGURE 26 Stereocontrolled synthesis of D-(-)-norgestrel.

compound 59 via the α-methylene ketone[92] reacts with the anion prepared from ethyl 7,7-ethylenedioxy-3-keto-octanoate (60) and sodium hydride to give the ester (61) in high yields. The crude ester is cyclized, hydrolyzed, and decarboxylated. The tricyclic compound (62) was isolated as an oil (see Hajos et al.[92]). Subsequent hydrogenation, cleavage of ethylene ketal, and cyclization produce 13β-ethyl-19-norandrostenedione (63) in an overall yield of 35 percent from (+)-enedione (57). D-(-)-norgestrel can be obtained from 63 by ethynylation with lithium acetylide in pure ethylenediamine. It is found that 13β-ethyl-17-ketone (63) is considerably less reactive than its 13-methyl counterpart toward acetylenic nucleophiles. The difference is attributed to the additional steric hindrance provided by the ethyl group.

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Microbial Formation of Therapeutically Valuable Steroids

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INTRODUCTION

Many steroids and their derivatives possess remarkable anti-inflammatory, anabolic, diuretic, antineoplastic, progestational, and androgenic properties. They are also effective in allergic, dermatologic, and ocular diseases, and in cardiovascular therapy. The synthesis of orally active progestogens led to their use as regulators of menstrual disorders in 1957 and to their application as oral contraceptives in 1960. They are also used in animal husbandry as fattening agents and for synchronizing estrus in farm animals. Their production is a $4 billion worldwide business (Lenz, 1983).

Interest in these compounds increased dramatically in 1949 when it was discovered that cortisone acetate alleviated the symptoms of rheumatoid arthritis (Hench et al., 1949, 1950). A sudden surge in the demand for this compound resulted, and it was hailed as a miracle drug for the treatment and cure of millions of sufferers of this crippling disease.

Cortisone was produced at that time from deoxycholic acid obtained from cattle bile by a laborious process consisting of 32 chemical steps (Sarett, 1949; see Figure 1). This process was improved, however, and presently Roussel Uclaf in France uses deoxycholic acid for the manufacture of cortisone by total synthesis.

Among the raw materials used to manufacture cortisone and its derivatives, diosgenin from barbasco, the Mexican yam, first dominated the world market. It has been estimated that between the mid-1950s and early 1960s well over 50 percent of all steroids manufactured worldwide originated from Mexican diosgenin (Djerassi, 1976). Diosgenin is almost ideal for the synthesis of not only corticosteroids but also 19-nor steroids and the diuretic spironolactone. The demand for this raw material continued to grow through the 1960s, but the situation began to change. Supplies began to decrease from overharvesting and rising prices as the Mexican government began to control collection of the yam. In 1975, yam collection was nationalized completely. Diosgenin is presently produced in the People's Republic of China for the synthesis of 19-nor steroid oral contraceptives and corticosteroids. It is also produced in Guatemala, Costa Rica, and India.

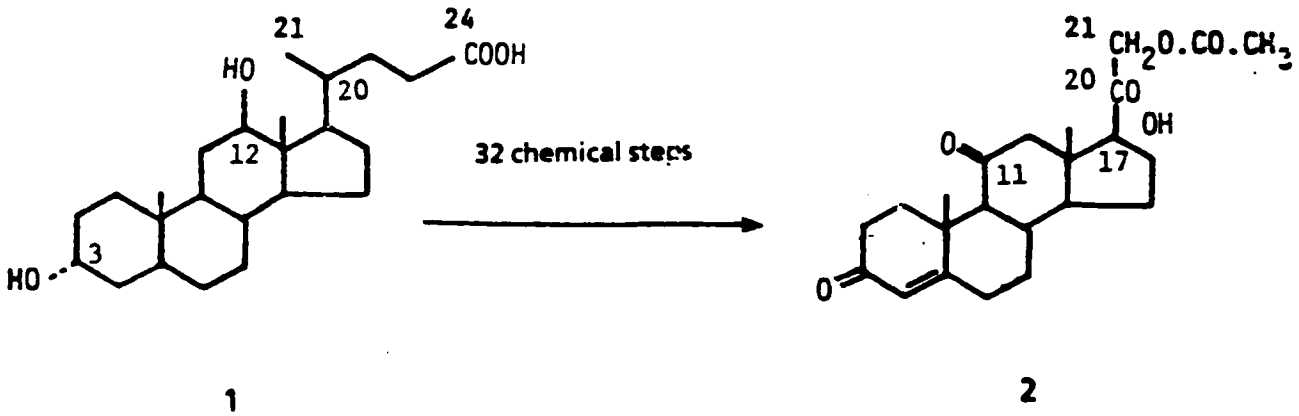

FIGURE 1 Synthesis of cortisone acetate (2) from deoxycholic acid (1).

As a result of the situation in Mexico, other raw materials also used in steroid manufacture began to assume new importance as alternatives to diosgenin. They include: stigmasterol from soybean oil (United States), hecogenin from sisal (East Africa, Ethiopia, Haiti), solasodine from Solanum plants (Ecuador, New Zealand, and Australia), and bile acids (France, the Netherlands). With the more recent elucidation of the bacterial side chain degradation of cholesterol and β-sitosterol, these compounds became extremely attractive for steroid manufacture as they are readily available in large amounts and inexpensive. Cholesterol is obtained as a by-product primarily from wool grease in Japan, and β-sitosterol (in a mixture with campesterol) from soybean oil in the United States and West Germany. Steroid hormones are also manufactured by total synthesis in France, Switzerland, East Germany, West Germany, Hungary, and the People's Republic of China.

MICROBIAL TRANSFORMATIONS OF STEROIDS

Hydroxylation

In the synthesis of corticosteroids such as cortisone or prednisolone, one of the more difficult steps was the stereospecific introduction of functional groups into the intermediates, and in particular the introduction of oxygen into the crucial C-11 position. This problem was solved in 1952 when it was reported that selected fungi of the order Mucorales (such as Rhizopus nigricans) carried out

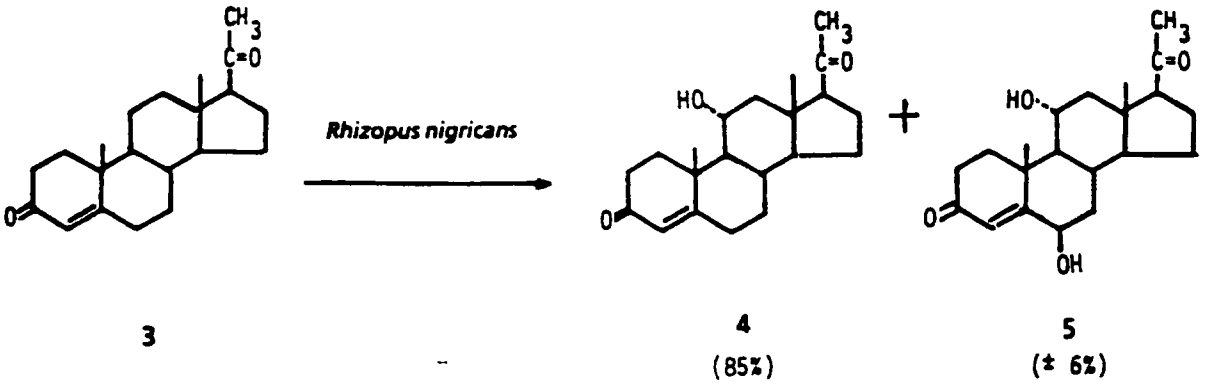

FIGURE 2 Fungal 11β-hydroxylation of progesterone (3) to 11α-hydroxyprogesterone (4) and 6β,11α-dihydroxyprogesterone (5).

the desired oxygenation with remarkable specificity and in high yields. Thus, progesterone was converted in one fermentation step to 11α-hydroxyprogesterone, the intermediate in the synthesis of cortisone and other corticosteroids (Peterson and Murray, 1952; Murray and Peterson, 1952; see Figure 2). Subsequently, other fungi such as Aspergillus ochraceus were also found to catalyze this reaction, and processes were developed in which the 11α-hydroxylated product was formed in 70-90 percent yields (with small amounts of 6β,11α-dihydroxyprogesterone; see Figure 2), at substrate concentrations in excess of 20 g/l (Abd-Elsamie et al., 1969; Hanson and Maxon, 1965.)

Oxygen was introduced into the strategic C-11 position also through 11β-hydroxylation (Figure 3) by means of other fungi such as Curvularia lunata and Cunninghamella blakesleeana. In this way, 11-deoxycortisone (cortexolone, Reichstein's Substance S), the preferred substrate in such a case, was transformed into hydrocortisone (cortisol, compound F) in approximately 60 percent yields with an additional 14α-hydroxylated by-product (Shull and Kita, 1955).

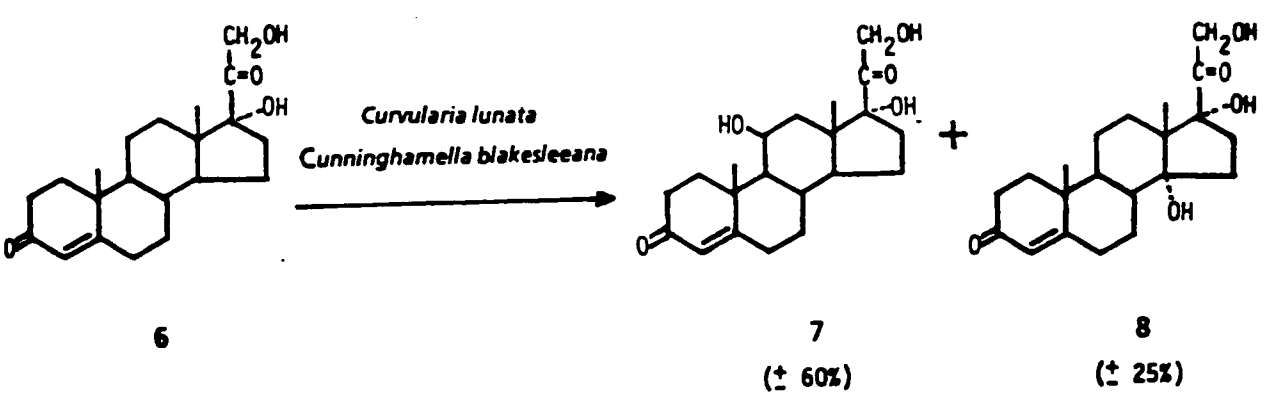

FIGURE 3 Fungal 11β-hydroxylation of Reichstein's Substance S (6) to hydrocortisone (7) and 14α-hydroxy-11-deoxycortisone (8).

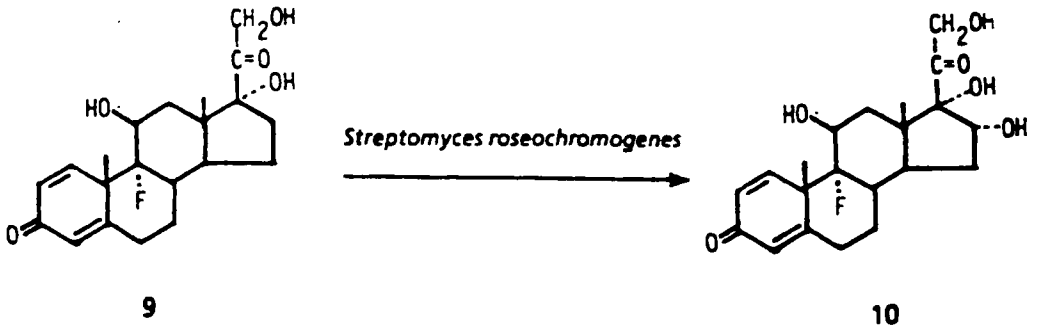

FIGURE 4 Microbial 16α-hydroxylation of 9α-fluoroprednisolone (9) to 16α-hydroxy-9α-fluoroprednisolone (triamcinolone, 10).

The anti-inflammatory activity of some corticosteroids such as hydrocortisone was further improved by the chemical conversion to 9α-fluorohydrocortisone, but this modification also caused an increase in salt retention. This undesirable side effect was reduced to some degree by microbial 1-dehydrogeneration of the latter compound to 9α-fluoroprednisolone (see the following section). When the resulting 9α-fluoroprednisolone was in turn hydroxylated at the 16α-position, it yielded 16α-hydroxy-9α-fluoroprednisolone (triamcinolone; see Figure 4). This compound is a potent anti-inflammatory steroid and essentially devoid of mineralocorticoid activity (Goodman and Smith, 1960, 1961).

Of other microbial reactions, 17α- and 21-hydroxylations by fungi may be functional, but they have been replaced by superior chemical routes.

To date, all the available carbons of the steroid molecule have been hydroxylated by selected microorganisms, but only the three mentioned above have found practical applications. Some of these reactions are also catalyzed by mammalian tissues (Table 1), but they are not of practical value since they would be quite expensive and difficult to perform on a large scale.

TABLE 1 Hydroxylations Carried out by Microorganisms and Mammalian Tissues

Microorganisms			Mammalian Tissues	
1α	7 α	15 α	1α	12 α
1β	7 β	15 β	2α	15 α
2α	9 α	16 α	2 β	16 α
2β	10 β	16 β	6α	17 α
3β	11 α	17 α	6β	18
5α	11 β	18	7α	19
5β	14 α	19	11 β	21
6β		21		

1-Dehydrogenation

Early in the development of these corticosteroids it was found that the anti-inflammatory property of some (such as cortisone and hydrocortisone) increases three to five times with the concomitant decrease in salt retention when a 1,2-double bond is introduced into their respective molecules. It was further discovered that this 1-dehydrogenation can be carried out quite conveniently and efficiently by means of several bacteria (*Arthrobacter simplex*, *Bacillus sphaericus*, *Nocardia restrictus*) and fungi (*Fusarium solani*, *Septomyxa affinis*).

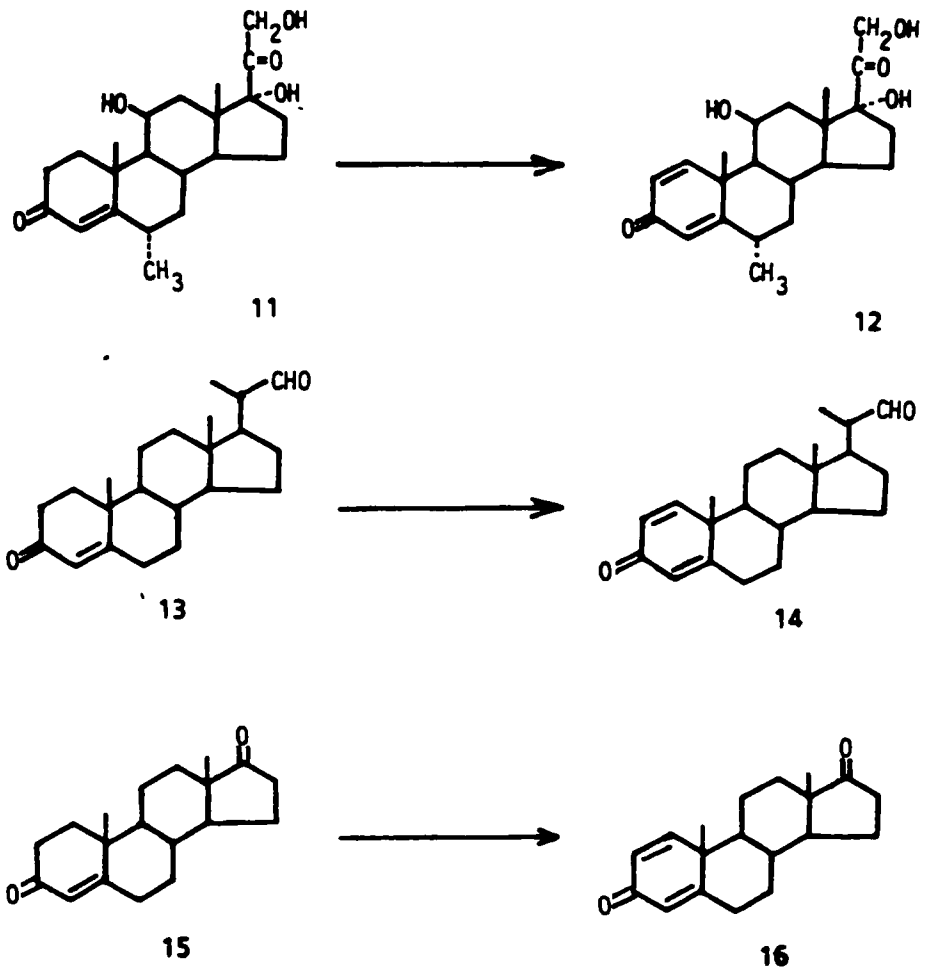

FIGURE 5 Microbial 1-dehydrogenation of selected steroids by *Septomyxa affinis*: 6α-methylhydrocortisone (11) to 6α-methylprednisolone (12); 3-oxobisnor-4-cholen-22-al (13) to 3-oxobisnor-1,4-choladien-22-al (14); and 4-androstene-3,17-dione (AD, 15) to 1,4-androstadiene-3, 17-dione (ADD, 16).

This microbial reaction has been incorporated into the processes by which several valuable 1-dehydrosteroids are produced: prednisone, prednisolone, 6α-methylprednisolone, 21-deoxy-9α-fluoro-6α-methylprednisolone, and triamcinolone (Figures 4 and 5). The same methodology is also used to dehydrogenate 4-androstene-3, 17-dione to 1,4-androstadiene-3, 17-dione, a valuable substrate for the chemical synthesis of estrone and 19-nor steroids (see below). Since the enzyme (1-dehydrogenase) that catalyzes this reaction is inducible, the addition of selected inducers (such as 3-ketobisnor-4-cholen-22-al or progesterone; see Figures 5 and 6) to the fermentation increases the efficiency of the reaction (Murray and Sebek, 1959; Koepsell, 1962).

Another technique, "pseudo-crystallofermentation," was described for converting 50 g of powdered cortisol/100 ml to prednisolone in five days. The product was recovered in 93 percent yields, and the procedure was developed into an industrial process (Kondo and Masuo, 1961).

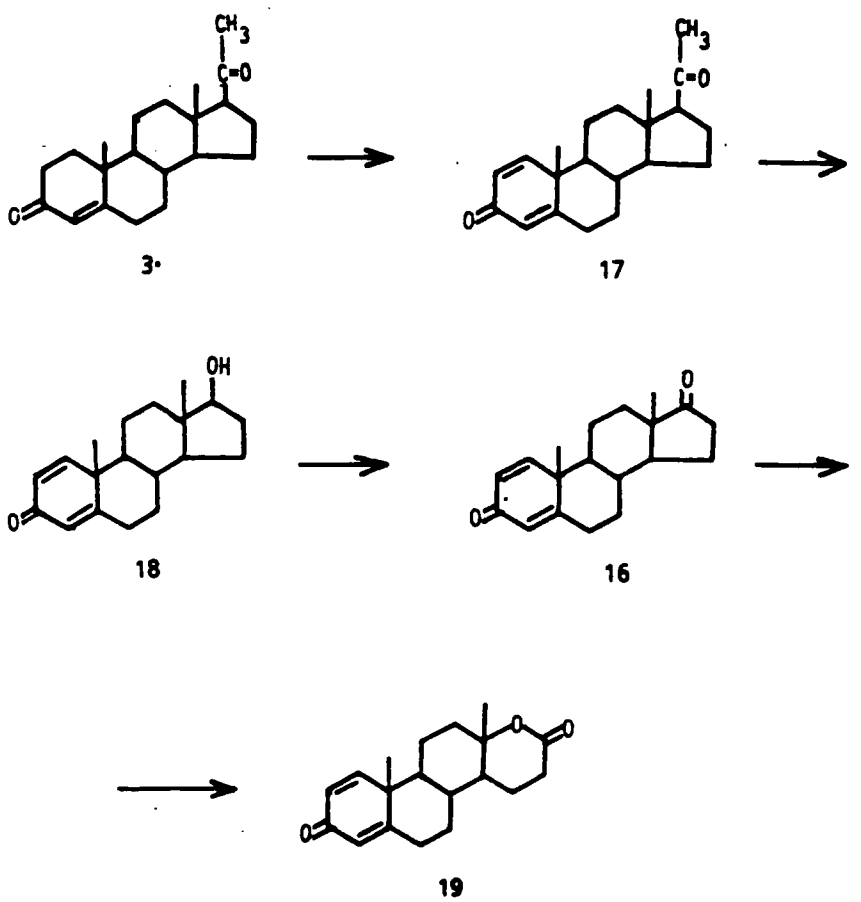

FIGURE 6 Metabolites of (3) produced by S. affinis: 1-dehydroprogesterone (17), 1-dehydrotestosterone (18), (16), and progesterone 1-dehydrotestololactone (19).

Undesirable By-products

Although the desired compounds are produced in high yields by the above processes, some by-products are also formed in varying amounts, thereby lowering the overall efficiency of the process.

Thus, for example, some of the microorganisms that carry out 1-dehydrogenation also cleave the side chain, oxidize the hydroxyl group at C-17, and carry out carbon-carbon scission of ring D (Figure 6).

Methods routinely used in fermentation development have been effective in reducing or eliminating such unwanted reactions. They include: selection of a suitable medium, adjustment and close control of fermentation conditions (length of incubation, aeration, temperature, addition of inhibitors), mutation, and strain selection.

MICROBIAL METABOLISM AND DEGRADATION OF STEROLS

The C-17 Side Chain

As indicated above, cholesterol and β-sitosterol have become attractive substrates for the production of pharmacologically active steroids. They are renewable raw materials, they are available in large quantities (approximately 100,000 tons worldwide in 1980; Lenz, 1983), and they are inexpensive. Cholesterol, which was first isolated from human gallstones, is extracted primarily in Japan from wool grease (wool imported from Australia and New Zealand) and fish oil, where it is present in 15 and 7 percent concentrations, respectively.

β-sitosterol is a component of soysterols (by-products of soybean oil processing) which consist of 21 percent stigmasterol, 49 percent β-sitosterol, and 27 percent campesterol (Itoh et al., 1973; see Figure 7). Of this mixture, only stigmasterol is used industrially in the United States for the synthesis of progesterone and other pregnanes (via 16-dehydropregnenolone acetate) because the C-22(23) double bond makes the side chain amenable to an efficient chemical cleavage. Since β-sitosterol and campesterol lack this double bond, chemical cleavage of their respective saturated aliphatic side chains is not economical as it also generates a number of undesirable by-products. For this reason they have been discarded as useless wastes.

More recently, however, ways were found (by selection of proper mutants, use of inhibitors, or chemical modification of the substrates) to allow side chain removal of these sterols without affecting their steroid nuclei. These observations were then developed into large-scale processing in which the side chains of cholesterol, β-sitosterol, and campesterol are cleaved in high yields specifically to 17-ketosteroids (4-androstene-3, 17-dione, 9α-hydroxy-4-androstene-3, 17-dione) in one fermentation operation (see below). Using additional microbial manipulation, conditions were also defined under which partial degradation of the steroid ring system occurs and yields other products useful as new substrates for further chemical syntheses.

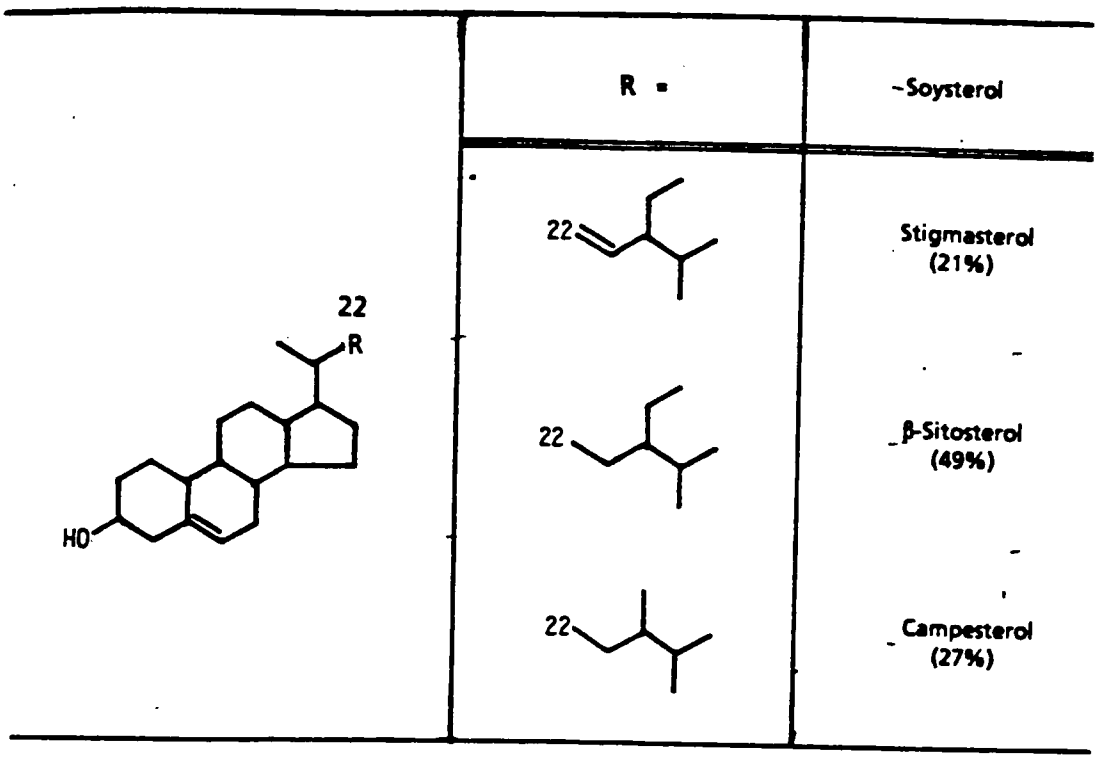

FIGURE 7 Soysterols extracted as by-products of soybean oil.

The origin of these developments can be traced back to 1913 when it was reported that mycobacteria were able to utilize cholesterol as the sole source of carbon for growth (Sohngen, 1913). This observation has since been confirmed many times, and bacteria belonging to the following genera were also found to grow on cholesterol: Arthrobacter (Corynebacterium), Azotobacter, Bacillus, Brevibacterium, Nocardia (Proactinomyces), Protaminobacter, Serratia, and Streptomyces (see Arima et al., 1969). Some bacteria were reported to degrade this substrate only partially.

Thus, methylheptanone was identified as a bacterial product originating from the cholesterol side chain (Horvath and Kramli, 1947; see Figure 8). In a related study the isolation of isocaproic, 3-oxo-4-etiocholenic, and Windaus' keto acids clearly showed that not only was the cholesterol side chain cleaved, its steroid ring structure was also metabolized (Turfitt, 1948; Stadtman et al., 1954). Subsequent data indicated that bacterial dissimilation of cholesterol can proceed via two different pathways: (1) by a stepwise degradation of the side chain, and (2) by a stepwise degradation of the steroid nucleus. The two pathways do not operate in sequence but proceed independently from each other. Thus, if degradation of the steroid nucleus does not take place, only the side chain will be degraded and vice versa.

Investigations of the mechanism of the cholesterol side chain degradation identified the individual enzymatic steps involved (Figure 9).

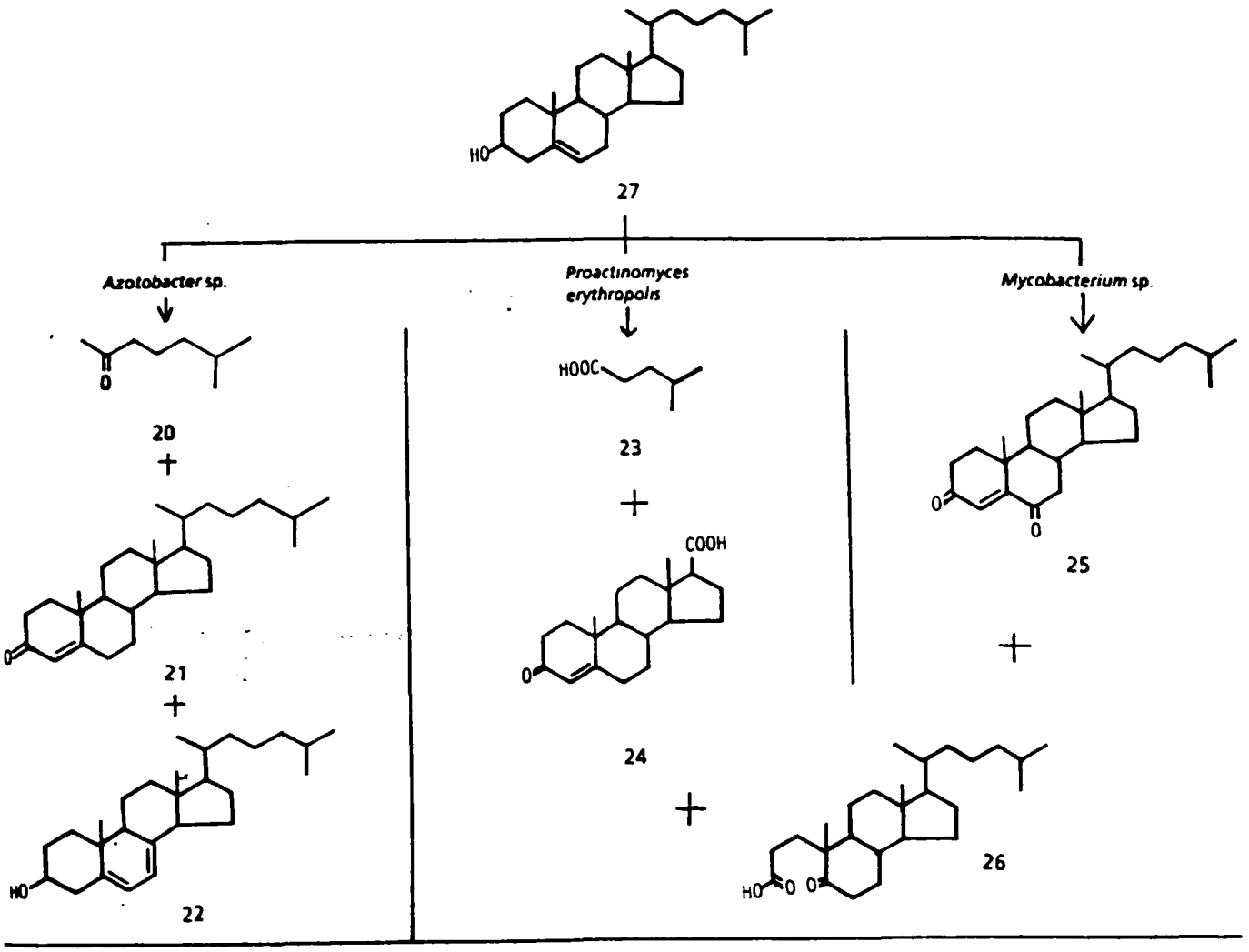

FIGURE 8 Earlier degradations of cholesterol (27) to methylheptanone (20), cholestenone (21), 7-dehydrocholesterol (22), isocaproic acid (23), 3-oxo-4-etiocholenic acid (24), cholestene-3,6-dione (25), and Windaus' keto acid (26).

The sterol is first converted to 4-cholesten-3-one (which may or may not be 1-dehydrogenated to 1,4-cholestadiene-3, 17-dione). The terminal C-26 methyl group is then hydroxylated and subsequently oxidized to the corresponding C-26 carboxylic acid. This acid in turn is degraded by a mechanism similar to the classical fatty acid β -oxidation, first to a C-24 carboxylic (3-oxo-4-cholen-24-oic) acid with a release of 1 mole of propionic acid, and then to a C-22 carboxylic (3-oxobisnor-4-cholen-22-oic) acid with the loss of 1 mole (see Sih et al., 1968; Arima et al., 1978; Iida et al., 1985). Upon removal of a second mole of propionic acid via a retroaldol reaction,

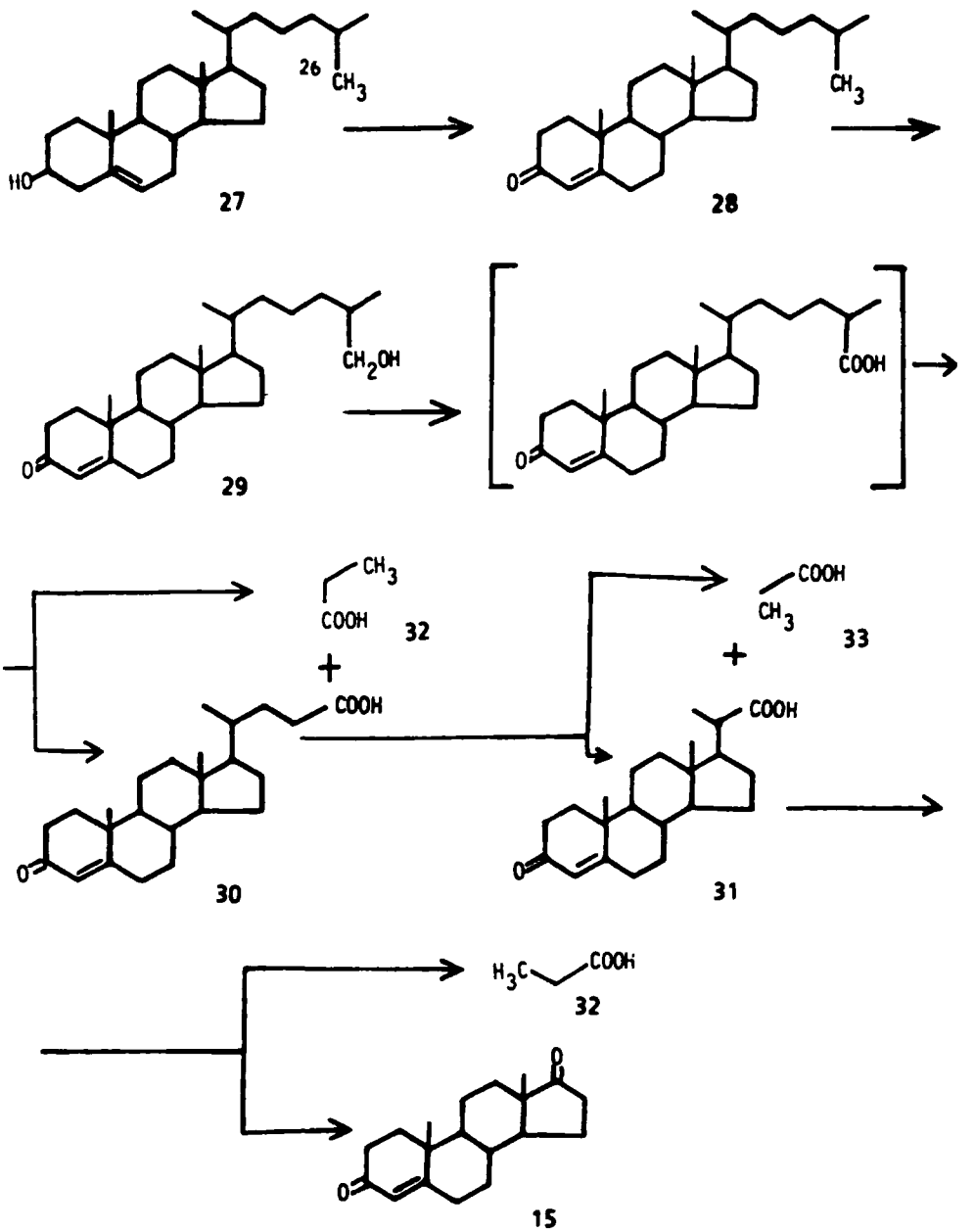

FIGURE 9 Microbial side chain degradation of cholesterol (27) to 4-cholesten-3-one (28), 26-hydroxy-4-cholesten-3-one (29), 3-oxo-4-cholen-24-oic acid (30), 3-oxobisnor-4-cholenic acid (31), (15), AD and propionic (32) and acetic (33) acids.

the C-22 carboxylic acid is then converted to a C-17 ketone (4-androstene-3, 17-dione, AD). If the substrate or any of the intermediates is 1-dehydrogenated, the final product of this sequence is 1,4-androstadiene-3, 17-dione (ADD).

The branched hydrocarbon side chain of β-sitosterol (and of campesterol, its 24-methyl analogue) is degraded by a similar oxidation sequence (see Figure 10). The process is again initiated by the hydroxylation of the C-26 methyl group, which in turn is oxidized to the corresponding carboxylic acid. The carbon-carbon fission at C-24-C-25 and C-24-C-28 of this acid results in the formation of 3-oxo-4-cholen-24-oic acid and 2 moles of propionic acid. As in the case of cholesterol this latter acid is further metabolized via 3-oxobisnor-4-cholenic-22-oic acid to AD (Fujimoto et al., 1982a, 1982b).

The Steroid Nucleus

Depending on the organism used, AD is then converted either to 9α-hydroxy-4-androstene-3, 17-dione (9α-OHAD) or 1,4-androstadiene-3, 17-dione (ADD; see Figure 11).

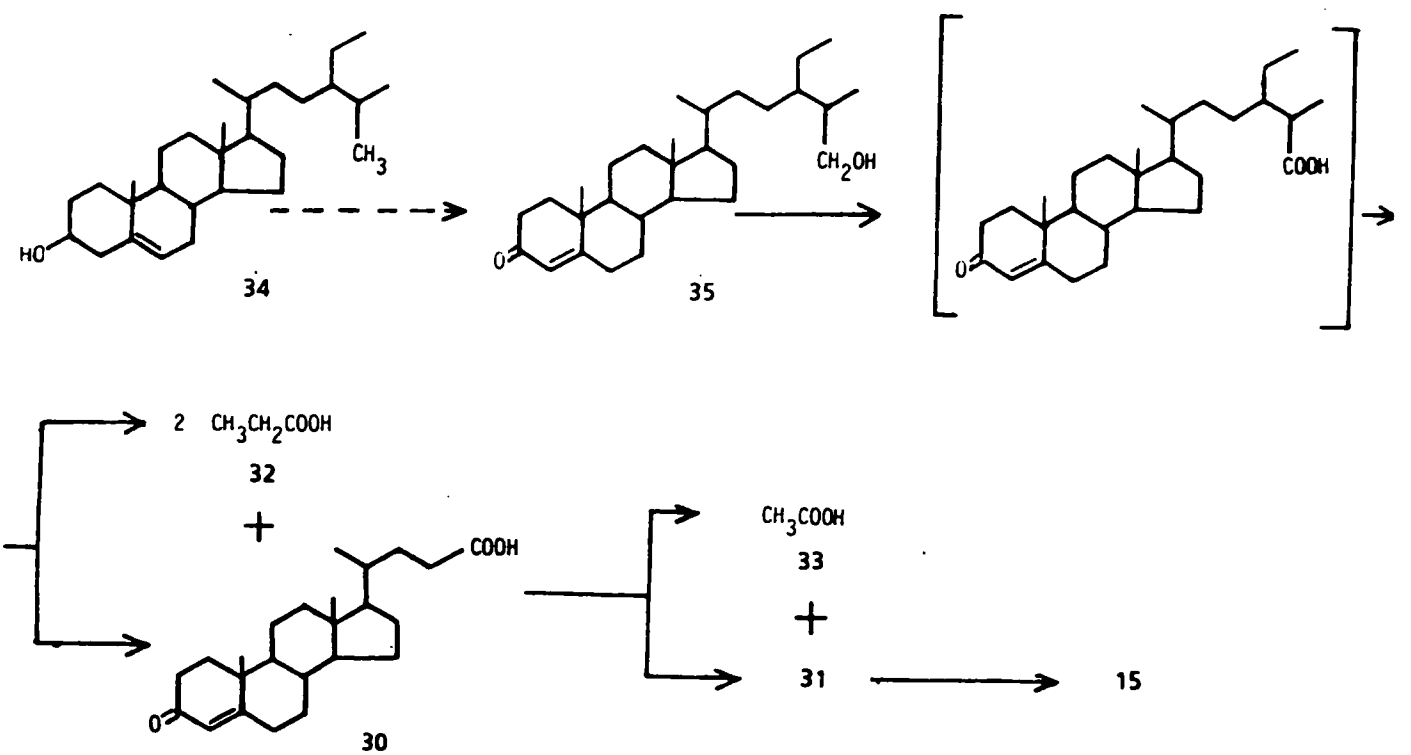

FIGURE 10 Microbial side chain degradation of β-sitosterol (34) to 24-ethyl-26-hydroxy-4-cholesten-3-one (35), 30, 31, 32, 33, and 15.

9α-OHAD is an excellent starting material for the synthesis of hydrocortisone acetate because its ring C is functionalized as the 9α-hydroxy group and the corticosteroid side chain can be easily added at C-17 by the known chemistry. Thus, by acid-catalyzed dehydration 9α-OHAD yields 4,9(11)-androstadiene-3, 17-dione, which is first converted to bromohydrin with the hydroxyl group at C-11 in the required α-configuration and is followed by the reductive removal of the bromine atom. The stereoselective introduction of the corticosteroid side chain at the C-17 position then results in the formation of hydrocortisone acetate (VanRheenen and Shephard, 1979). This synthesis thus offers an alternative to the functionalizing of the C-11 position by microbial hydroxylation. Its additional flexibility is that it also yields 17-hydroxyprogesterones, the starting materials for a number of therapeutically important antifertility agents (Shephard and VanRheenen, 1977).

The second product, ADD, is pyrolyzed in high yields to estrone from which 19-nor steroid oral contraceptives are synthesized by a modified Birch reduction.

In the subsequent steps of the steroid nucleus degradation (Figure 11), 9α-OHAD is dehydrogenated and ADD is 9α-hydroxylated. The product of both reactions is the same, 9α-hydroxy-1, 4-androstadiene-3,17-dione (9α-OHADD). Since it is highly unstable, 9α-OHADD immediately undergoes simultaneous aromatization of ring A and cleavage of ring B between C-9 and C-10 by a nonenzymatic reverse aldol-type reaction, and yields 3-hydroxy-9, 10-<u>seco</u>-1,3,5(10)-androstatriene-9, 17-dione (HSAD). Through a subsequent hydroxylation at C-4 (3,4-dihydroxy-9, 10-<u>seco</u>-1,3,5(10)-androstatriene-9, 17-dione, DHSAD), ring A is opened by a <u>meta</u> cleavage whereby a 2,6-dioxocarboxylic acid is formed. This acid is then cleaved to (1) 2-oxo-4-hydroxyhexanoic acid, which in turn yields pyruvic acid and propionaldehyde; and (2) hydrindene carboxylic acids, which are useful substrates in the chemical synthesis of retrosteroids. They are further metabolized to succinic acid and eventually oxidized to carbon dioxide and water (see Kieslich, 1985).

In addition to its theoretical significance, the elucidation of this degradation sequence was of considerable practical interest. It showed that (1) AD, 9α-OHAD, and ADD (convenient substrates in the synthesis of steroid drugs) are intermediates in these processes; and (2) they are formed only after the side chains of the respective sterol substrates have been completely removed. It also showed that their degradation is initiated only after they have been 9α-hydroxylated and 1-dehydrogenated, thereby yielding an unstable 9α-hydroxy-1, 4-dien-3-one, which initiates the decomposition of the steroid nucleus. If one or both of the enzymes involved (9α-hydroxylase and 1-dehydrogenase) is inactivated, degradation of the steroid nucleus is prevented and the respective products accumulate in the medium.

To achieve this accumulation three different methods were used: (1) the substrates were chemically modified; (2) the sterol degradation was performed in the presence of inhibitors of the two enzymes involved; and (3) the degradation was carried out by mutants lacking 9α-hydroxylase 1-dehydrogenase, or both.

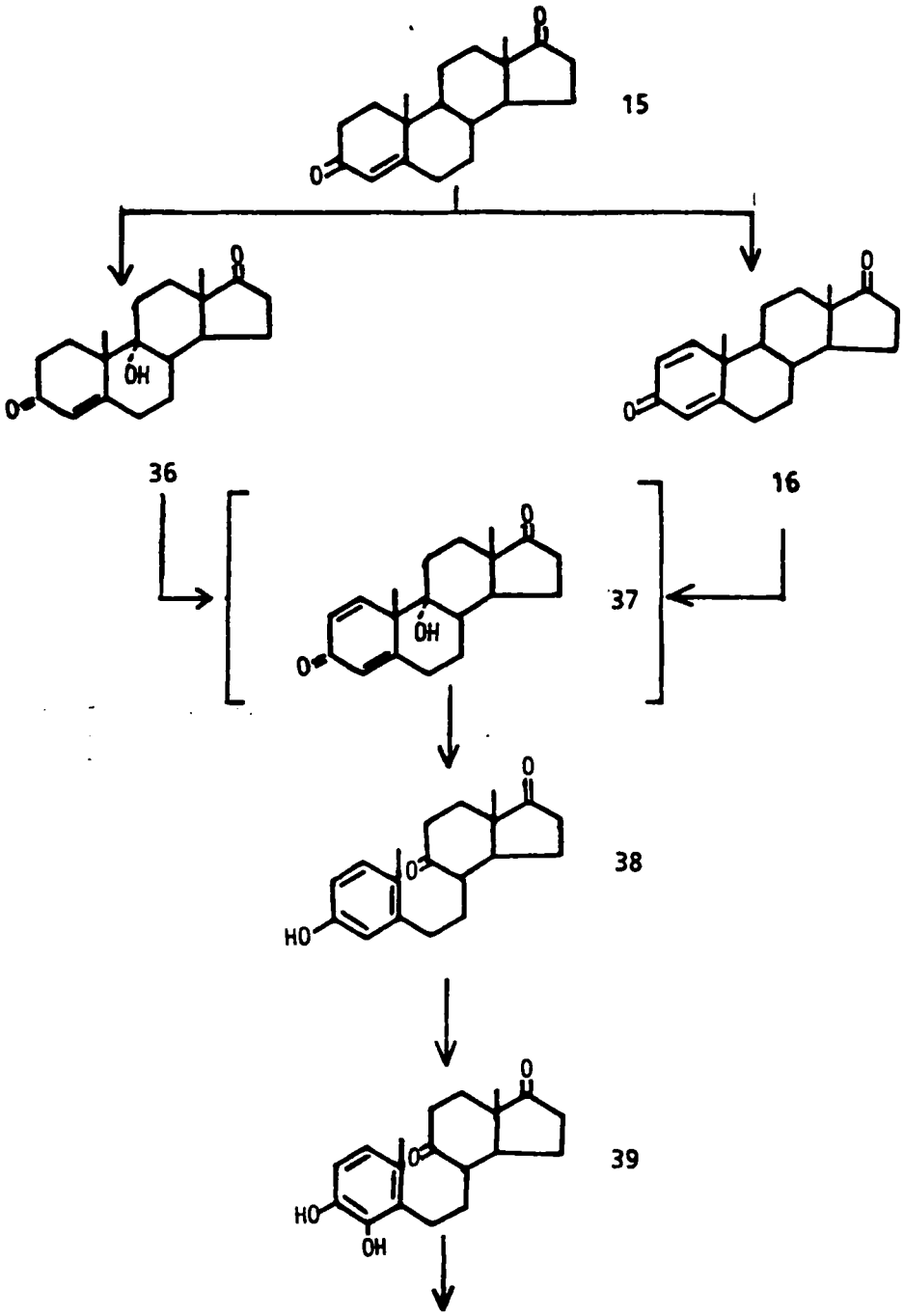

FIGURE 11 Microbial degradation of the steroid ring structure (4-androstene-3, 17-dione, 15) to: 9α-hydroxy-4-androstene-3, 17-dione (9α-OHAD, 36); 16, 9α-hydroxy-1, 4-androstadiene-3, 17-dione (9α-OHADD, 37); 3-hydroxy-9, 10-*seco*-1,3,5(10)-androstatriene-9, 17-dione (HSAD, 38); 3,4-dihydroxy-9, 10-*seco*-1,3,5(10)-androstatriene-9, 17-dione (DHSAD, 39).

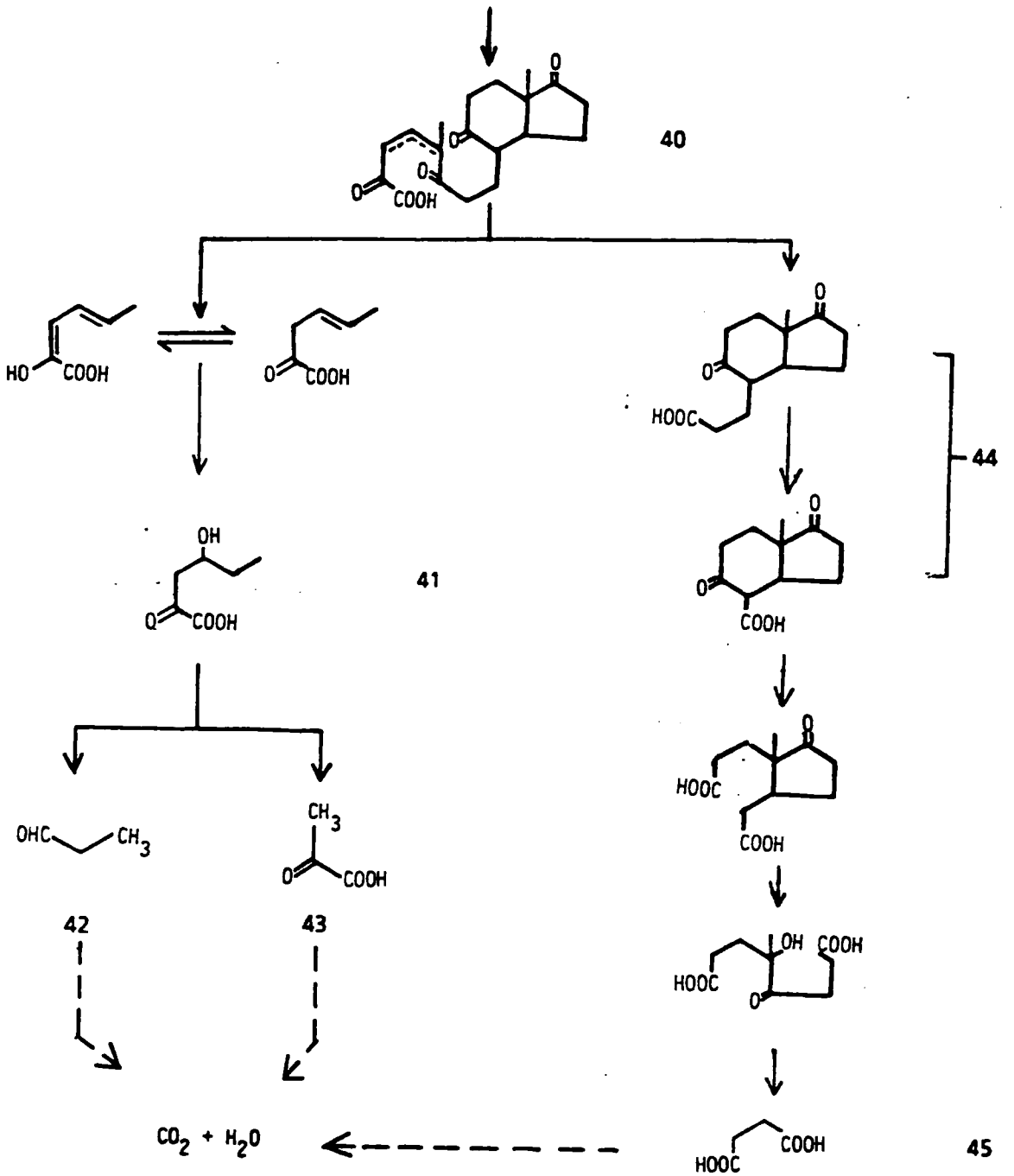

FIGURE 11 (continued) 2,6-dicarboxylic (40) and 2-oxo-4-hydroxyhexanoic (41) acids; propionaldehyde (42); and pyruvic (43), hydrindene carboxylic (44), and succinic (45) acids.

Method 1. The observation that degradation of the steroid molecule was prevented by modifying the structure of the substrate was first made in 1958. A pseudomonad was described to convert 19-hydroxy-4-androstene-3, 17-dione to estrone which was not further metabolized and accumulated in the medium (Dodson and Muir, 1958). This conversion was shown to be carried out by a strain of Nocardia restrictus as well (Sih and Rakim, 1963). In addition, estrone was formed from 19-hydroxy-4-cholesten-3-one, 19-hydroxy-4-sitosten-3-one (Sih and Wang, 1965), and 3β-acetoxy-19-hydroxy-5-cholestene (Sih et al., 1965). The latter conversion is efficient (72 percent yields) and of particular interest since this substrate is conveniently prepared from cholesterol acetate in three chemical steps (Figure 12).

A microbial reaction was also included in an efficient synthesis of 19-nor steroids. As in the synthesis of estrone, 3β-acetoxy-5-chloro-6,19-oxidocholestane (prepared from cholesterol acetate in two steps; see Figure 12) is converted to 6,19-oxido-4-androstene-3, 17-dione, a key intermediate in the synthesis of 19-nor steroids (Sih et al., 1965).

These combined chemical-microbial procedures represent the shortest and most efficient routes for the synthesis of estrone and also of 19-nor-17α-ethynyltestosterone and related contraceptive agents (see Djerassi, 1966).

Method 2. As noted earlier, steroid 9α-hydroxylase is a key enzyme responsible for the degradation of the steroid nucleus. Thus, inhibition of its action means that the integrity of the nucleus is preserved while the selective side chain degradation and 1-dehydrogenation proceed unimpaired and result in the formation and accumulation of ADD. Since the hydroxylase is a Fe^{2+} containing mono-oxygenase, the lipophilic iron chelating agents accomplish the desired selective side chain degradation. Among them, αα'-dipyridyl, 1,10-phenanthroline, and 8-hydroxyquinoline proved most effective for the accumulation of ADD. Others that were active by the same mechanism included cupferron, diphenylthiocarbazone, diethyldithiocarbamate, isonicotinic acid hydrazide, or o-phenylenediamine. Also effective were Ni^{2+}, Co^{2+}, Pb^{2+}, SeO_3^{2-}, and AsO_2^{1-} by replacing Fe^{2+} or blocking SH-functions.

On the basis of this and other information, fermentation processes were developed for the production of ADD from cholesterol in Japan (Mitsubishi Chemical Industry) and the Netherlands (Gist-Brocades). ADD in turn is a valuable substrate for the synthesis of a number of steroid hormones: estrogens (estradiol, estriol, mestranol, dimethisterone), progestogens (norethisterone, norethynodrel, ethynodiol diacetate), androgen (testosterone), anabolic (norethandrolone) and diuretic agents (spironolactone), as well as a corticosteroid (triamcinolone).

Method 3. The third method is superior to the two described above. To generate the above products, it requires neither modification of the substrate nor the addition of metal ions and

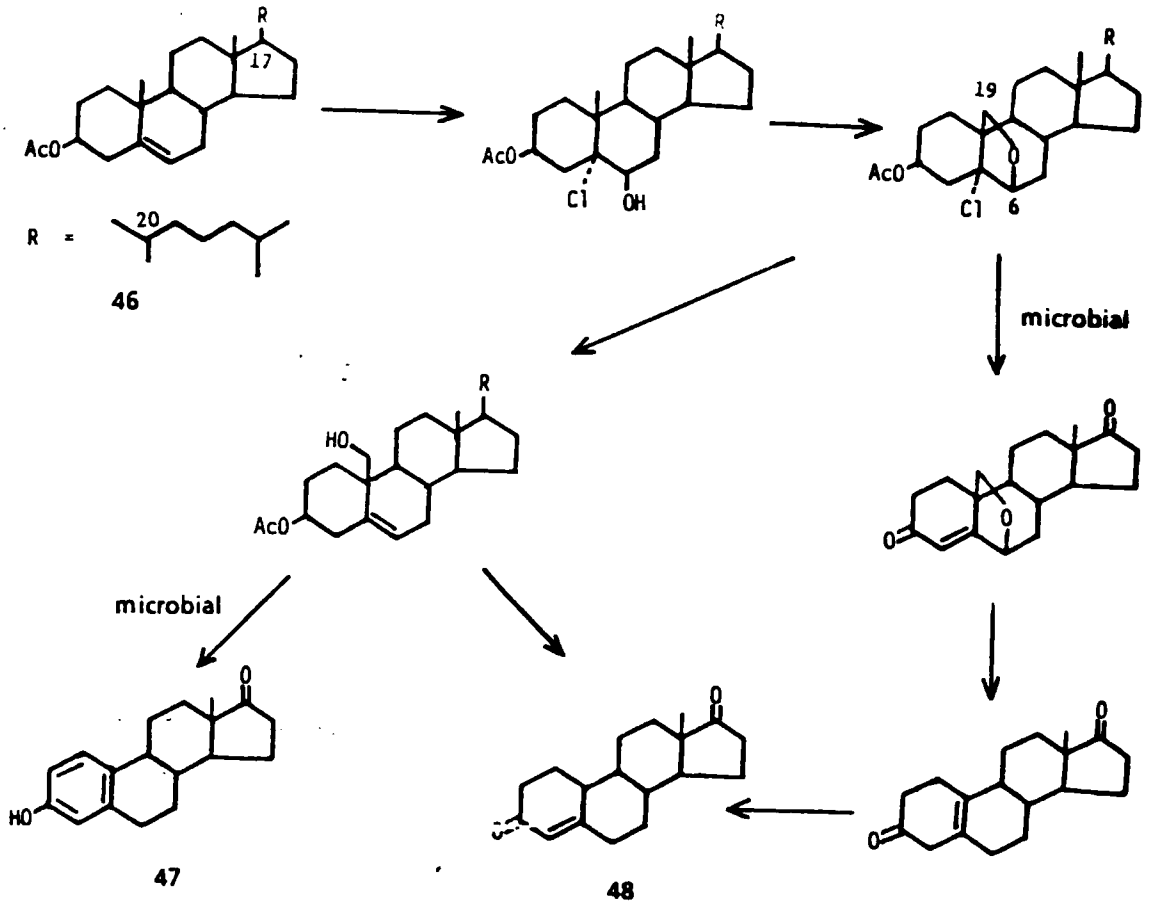

FIGURE 12 Synthesis of estrone (47) and 19-nor-4-androstene-3, 17-dione (48) from cholesterol acetate (46).

chelating agents to the fermentation. Rather, it employs mutants derived from the total sterol degraders which are selectively blocked in the decomposition of the steroid nucleus. In addition, other mutants may be selected from such mutated populations, which are blocked at various stages of the degradative pathway, making it feasible to isolate the respective intermediary products, including ring A-degraded tricyclic compounds.

The methodology of generating, selecting, and evaluating such mutants is a well-established process (see Marsheck et al., 1972; Wovcha et al., 1978.) It involves mutation of a potent sterol-degrading organism and selection of mutants with the desired properties. The biotransformations are generally carried out by growing the selected mutants in nutritionally rich media in shaken flasks and in aerated fermentors. When the late logarithmic or early stationary growth phase is reached, the substrate is added, the incubation is continued, and the progress of the biotransformation is monitored by appropriate analytical methods. Because of their

inherently poor solubilities in water, the steroid substrates may be added to the medium in fine suspensions or dissolved in water-miscible organic solvents (N,N-dimethylformamide, acetone, dimethyl sulfoxide, ethylene glycol, and ethanol). Given their toxicity, the concentrations of these solvents should be as low as possible. To eliminate such toxic effects, finely powdered substrates are also used or water-soluble derivatives prepared (cycloborate esters, steroid 21-hemisuccinates). When the bioconversion is completed, the products (and any unreacted substrate) are isolated by the established solvent extraction procedure.

Although these transformations have been carried out by the traditional batch fermentations, they can also be performed with washed resting cells, spores, or isolated enzymes which have been immobilized on a suitable carrier (DEAE-cellulose, acrylamide) by the cross-linking immobilization technique. The advantage of this manipulation is a minimal loss of enzyme activity and improved stability.

CONCLUSION

Microorganisms are important in the manufacture of various compounds such as antibiotics, organic and amino acids, vitamins, nucleotides, and nucleosides. They have also been used with considerable success as catalysts of specific reactions with which some industrially valuable compounds are produced: hydrolysis of penicillins G and V for the production of superior semisynthetic penicillins; preparation of pure L-amino acids by resolution of their racemic DL-mixtures; oxidation of D-sorbitol to L-sorbose and formation of 2-ketogulonate from glucose in the synthesis of vitamin C; and reactions involved in the synthesis of the artificial sweetener aspartame.

In the synthesis of steroid hormones two kinds of microbial manipulation had a considerable impact on the economic production of these compounds: 11β- and 11α-hydroxylations and 1-dehydrogenation in the early 1950s, and the selective and efficient side chain degradation of abundant and inexpensive sterols in the 1960s and 1970s.

In view of the effectiveness and established therapeutic value of the key steroid hormones, one might expect that further improvements will be made in their production by means of new and more efficient microorganisms. This effort will also include modern methodologies such as chemostat mutation, protoplast fusion, and recombinant DNA technology.

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APPENDIXES

APPENDIX A

Keynote Address

Didin S. Sastrapradja
Assistant (II) Minister of State
for Research and Technology

It is a pleasure and honor for me, on behalf of the Minister of State for Research and Technology and Chairman of the steering committee, to welcome you to the opening of this Workshop on Biotechnology of Steroid Compounds as Contraceptives and Drugs. I would also like to extend our warm greetings to the participants from the United States, as well as from Indonesia, who will share their invaluable knowledge, experience, and ideas in the deliberations and discussions at this workshop.

In October 1983, the U.S. National Research Council and the Indonesian Ministry of State for Research and Technology convened a symposium in Washington, D.C. on potential Indonesia-U.S. collaboration in science and technology. One of the priorities recommended by the symposium was developing cooperative programs in the field of biotechnology and related subjects.

As a follow-up to the symposium, two workshops were organized by the U.S. National Research Council and the Indonesian National Research Council in Jakarta: the Workshop on Marine Algae Biotechnology, December 11-13, 1985, and the Workshop on Biotechnology in Agriculture, March 13-14, 1986. At the first workshop various aspects of the cultivation, processing, and marketing of marine algae were discussed. At the second workshop the discussions focused on animal production, with an emphasis on embryo transplantation, plant cell and tissue culture, biological nitrogen fixation, and bioconversion of agricultural wastes.

This workshop, the third in the series, will deal with the production of sitosterols from agricultural by-products and other natural resources, the production of steroid compounds by plant cell and tissue culture as well as by fermentation, and chemical synthesis. It is also expected that the discussions will cover the establishment of research and development programs, scientific and technical manpower training, industrial applications, feasible U.S.-Indonesia cooperation in this field, and other topics required for the plan of action.

About 45 persons were invited to this workshop--five from the United States and 40 from Indonesia. They come from various government research and development institutes, universities, and state enterprises. According to the most recent information, 30 Indonesian participants are present this morning.

We are aware that Indonesia is a newcomer in the field of modern biotechnology, and that there is a long way to go before we can actively utilize it to help advance our industry. Therefore, it is our sincere hope that this workshop will produce tangible results that can be used to speed up the development of biotechnology in Indonesia.

In conclusion, may I take this opportunity to officially extend our appreciation to the U.S. National Research Council for its continuous assistance and cooperation, to the U.S. Agency for International Development, to the distinguished participants, and to the Office of the Minister of State for Research and Technology, the Agency for the Assessment and Application of Technology, the secretary and staff of the Indonesian National Research Council, the organizing committee, and others who in one way or another made this workshop possible.

Last but not least I wish you all every success in your discussions and deliberations and may your stay in Jakarta be fruitful and enjoyable.

APPENDIX B

Opening Remarks

Margaret Bonner
Acting Director,
USAID Mission in Indonesia

The U.S. Agency for International Development [USAID] is pleased to be associated with the third biotechnology workshop jointly presented by the U.S. National Research Council [NRC] and the Indonesian National Research Council [DRN] for several reasons. One reason is the role that USAID plays in promoting the continuing relationship between the NRC and DRN. We believe that this is an important relationship which serves the purpose of bringing together some of the top scientists from our respective countries. This is important from both a micro and a macro perspective. On the micro side it is significant in that the scientists of both countries can share the latest in technological and scientific advancement with the potential relative benefits that might flow from such an exchange. The relationship is also important in a macro sense in that establishment of strong ties between Indonesia and the United States in the scientific field has an influence in strengthening the existing friendship between our countries.

When I first heard that steroids were to be the main topic of discussion at this workshop, I had an image of athletes, as we say in the United States, "pumping iron," since as a lay person the only knowledge I had of steroids was its connection with building athletic capacity. I found it puzzling that this topic had been chosen, but I assumed that our two illustrious scientific organizations were not going to be involved in pumping iron. And I was proven correct. This workshop is not concerned with producing super human athletes. Rather, it will affect an area of great interest to USAID and one in which we have had a long involvement in Indonesia--family planning.

The success of Indonesia's family planning program is known worldwide. Through its determined efforts, the crude birth rate decreased from 46 per 1,000 population in 1970 to 33 per 1,000 in 1984. Success also brings problems, however, and Indonesia's rapidly expanding family planning program requires an increasing supply of contraceptives, including oral ones. The distribution of these has increased from approximately 1-2 million cycles in 1970 to over 65 million cycles in 1985. With the current anticipated population growth, and with over 25 million women in the childbearing age group, it is estimated that over 150 million cycles will be needed by the year 2000.

Steroid contraceptives and drugs can now be produced industrially from sitosterols through fermentation. Moreover, sterols as raw materials are relatively inexpensive because they can be obtained as

by-products from a number of agricultural products and from tropical plants. Considering the importance of steroid contraceptives in the national family planning program as well as the high value of steroid drugs, we are pleased to support Indonesia's efforts in producing steroids from indigenous natural products. Furthermore, the implication of Indonesia becoming possibly self-sufficient in the production of steroid contraceptives by utilizing indigenous natural resources fits the government's policy of reducing imports and strengthening domestic production capabilities.

USAID is proud to be a part of this association. We would like to congratulate both the U.S. National Research Council and the Indonesian National Research Council on past successes from earlier workshops and to wish you a fruitful exchange in this workshop on the production of steroid compounds for contraceptives and drugs.

APPENDIX C

Closing Remarks

Didin S. Sastrapradja

We have all listened carefully and discussed extensively the reports of the working groups and finally heard the report of the steering committee. After three days of serious discussions on various aspects of steroid compounds as contraceptives and drugs, we now come to the end of our workshop. I am very impressed with the results of this meeting in which all of you have successfully formulated the potential sources of steroid compounds and methods of production. I hope that the results of this meeting can be used as a basis for the development of a detailed plan of action for implementation.

As I explained in the opening session, Indonesia is paying serious attention to the development of a national capability in biotechnology. The Office of the Minister of State for Research and Technology is working hard to formulate "a development strategy on biotechnology" and is trying to identify priorities for action. I am therefore convinced that the ideas formulated in this workshop will contribute significantly to the completion of the strategic policies that will lead to implementation.

On behalf of the Minister of State for Research and Technology, I wish to extend our appreciation to all of you for your participation in this meeting, and to the U.S. National Research Council, and particularly to Mrs. Rose Bannigan, for its continuous cooperation and assistance.

APPENDIX D

Workshop Agenda

DECEMBER 15, 1986

08.30-09.00	Registration
09.00-09.30	Opening Ceremony Keynote Address Prof. Dr. Didin S. Sastrapradja Assistant (II) Minister of State for Research and Technology Opening Remarks Dr. Margaret Bonner Acting Director, USAID Mission in Indonesia
09.30-09.45	Coffee Break
09.45-10.45	Presentation: Development of Steroid Compounds as a Raw Material for Drugs, by Drs. Utarto Chairperson, Dr. Susono Saono Rapporteurs, Ir. Sadjuga and Dra. Donowati
10.45-12.00	Presentation: Extraction and Biotransformation Studies of Steroids and Morphinan Alkaloids from Indonesian Biological Resources, by Ischak Lubis, M. Pharm. and Dr. Susono Saono Chairperson, Dr. Ponis Tarigan Rapporteurs, Dr. Hani Mochtar and Dra. Donowati
1.00-14.45	Presentation: The Role of Plant Tissue Culture in Pharmacy and Biotechnology, by Dr. E. John Staba Chairperson, Dr. Gustaaf A. Wattimena Rapporteurs, Ir. Sadjuga and Dra. Donowati

14.45-15.45	Presentations: Production of Steroid Compounds by Fermentation, by Dr. O. K. Sebek Synthesis of Steroid Oral Contraceptives Available in the United States, by Dr. Hyun K. Kim Chairperson, Dr. Ponis Tarigan Rapporteurs, Ir. Hasni Mochtar and Dra. Ratna Chandra
15.45-16.00	Coffee Break
16.00-17.00	Groups Discussion (Breaking into three groups)

DECEMBER 15, 1986

09.00-10.30	Groups Discussion (Indonesian paper presented in each group)
10.30-11.00	Coffee Break
11.00-12.45	Groups Discussion (continued)
12.45-13.30	Lunch
13.30-15.00	Groups Discussion (continued)
15.00-15.30	Coffee Break
15.00-17.00	Groups Discussion (continued)
15.30-17.00	Groups Discussion (continued) Formulating conclusions and recommendations of groups

DECEMBER 17, 1986

09.00-10.30	Plenary Session Chairperson, Drs. Utarto Rapporteur, Drs. Taufiq Amin Notulists, Drh. Ida Kusumah and Dra. Ratna Chandra
11.00-12.00	Coffee Break
12.00-13.00	Closing Ceremony - Report of the Steering Committee - Comments on the Workshop by Dr. Monroe E. Wall, Chairman of NRC Panel - Remarks, Prof. Sediono Tjondronegoro, Secretary, Indonesian National Research Council

- Closing Remarks, Prof. Dr. Didin S. Sastrapradja, Assistant (II) Minister of State for Research and Technology

13.00-14.00 Lunch

APPENDIX E

Workshop Participants

STEERING COMMITTEE

Didin S. Sastrapradja
A. M. Satari
Sediono M. P. Tjondronegoro
Qei Ban Liang
Utarto
Susono Saono
Haryanto Dhanutirto
Rose Bannigan

WORKING GROUPS

Sitosterol Sources from Agricultural By-products and Natural Resources

Kosasih Padmawinata, Chairman
Ischak Lubis, Rapporteur
P. Soedigdo
Sutaryadi
Soekeni Soedigdo
Padmono Tjitroreksoko
Arini
Sadjuga
Monroe E. Wall
Rose Bannigan

Production of Steroid Compounds by Plant Cell and Tissue Culture

Gustaaf A. Wattimena, Chairman
Livy Winata Gujnawan, Rapporteur
E. Noehardi
Usep Sutisna
Gunawan Indrayanto
Untung Suwahyono
Muchamad Sholichin
Puspa Tjondronegoro
E. John Staba
Hasni Mochtar
Donowati

Production of Steroid Compounds by Fermentation and Chemical Synthesis

Oei Ban Liang, Chairman
Taufiq Amin, Rapporteur
Ponis Tarigan
Susono Saono
Triadi Basuki
Sardjoko
Utarto
Ibrahim Sastramihardja
Saraswati
Haryanto Dhanutirto
Hyun K. Kim
Oldrich K. Sebek
Ida Kusumah
Mawarwati
Ratna Chandra

ORGANIZING COMMITTEE

Haryanto Dhanutirto, Chairman
Jana Anggadiredja, Secretary
Sawedi
Dadang A. Permadi
Erni Titi Marpaung
Purwandoko
Sukmaya
Budi Minerva
Ratna Wulan Karang
Asti Suryani
Yessy Mudjiati
Indang Wahyurini

OTHERS

Margaret Bonner
Carol Carpenter
David Christensen
Hardijono
Donathus Pakpahan
A. B. Van Rennes
Edi Setianto
Mochtar Machful
Moch Mochtar

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Workshop on biotechnology of steriod compounds as contraceptives and drugs.

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