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Review

Testolactone: The Rise and Fall of a Drug

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Abstract: Testolactone is structurally related to testosterone and belongs to the first generation of aromatase inhibitors. It is a non-selective irreversible aromatase enzyme inhibitor that was one of the first steroids used in the clinical treatment of breast cancer. The use of testolactone in the treatment of breast cancer started in 1970, although its ability to inhibit aromatase was not discovered until 1975. Its use was primarily based on the inhibition of estrogen synthesis, which was applied in the treatment of estrogen-dependent breast cancers, in the treatment of disorders of sex steroid excess, familial male-limited precocious puberty, or in the treatment of patients with McCune–Albright syndrome, etc. The weak inhibitory activity of testolactone, and the moderate clinical response, prevented its widespread use, which ultimately resulted in withdrawal from the drug market in 2008. This review paper is dedicated to testolactone, its rise in the second half of the 20th century, and its fall in the first decade of the 21st century. Regardless of withdrawal from the market, for many years testolactone was a drug that improved the quality of life of patients facing one of the most serious diseases today, and for this reason, this paper describes medicinal application, synthesis, and modifications of testolactone.

Keywords: Δ^1-testololactone; teslac; aromatase inhibitor; breast cancer; precocious puberty; gynecomastia

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1. Introduction

Cytochrome P450 aromatase is an enzyme responsible for the aromatization of A ring in androgens, and, thus, biosynthesis of estrogen hormones, controlling the levels of androgens and estrogens in the human organism. Inhibition of its activity is one of the predominant courses of treatment of estrogen-dependent illnesses. One of the most frequent estrogen-dependent illnesses is breast cancer, which is also the most frequent cancer in women. The first synthetic steroid compound with clinical application in the treatment of estrogen-dependent breast cancer is testolactone. Although it was in clinical use from 1970 to 2008 [1], the mechanism of action through the inhibition of enzyme aromatase was discovered during its medical application [2]. Since this compound was the first steroid used as an aromatase inhibitor (AI) and one of the first AI in clinical application, it was classified as a first-generation AI. Testolactone was administered orally or as an intramuscular injection for the treatment of breast cancer, precocious puberty, and gynecomastia. Besides testolactone and the trade name Teslac, this compound can also be found under the names: Δ^1-testololactone, 1-dehydrotestololactone, 17a-oxa-D-homo-1,4-androstadiene-3,17-dione, and 13-hydroxy-3-oxo-13,17-secoandrosta-1,4-dien-17-oic acid δ-lactone. Its structure is characterized by an androgenic steroid core with a six-membered D-lactone ring in place of a five-membered carbocyclic D-ring, and 1,4-diene-3-one moiety in A-ring (Figure 1). Although clinical application was discontinued because new, more potent drugs emerged, research regarding its activity in the treatment of other medical conditions continues to this day. Furthermore, the structure of testolactone is often used as a starting point in the design of new more biologically active steroid compounds. These facts led us to the idea of writing a review focused solely on testolactone, since, to this

day only one was published [3], and the rest were dedicated to aromatase inhibitors in general [4–9].

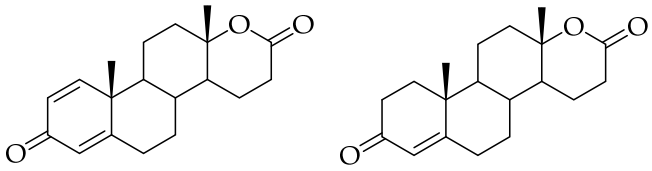


Figure 1. Structures of testolactone (**1**) and testololactone (**2**).

2. Literature Research Analysis

Literature regarding testolactone was retrieved from five online databases: PubMed, Scopus, Web of Science, ScienceDirect, and Google Scholar, during October and November 2022. A primary search was carried out using "testolactone" as a search term. When the search was performed on 18th November 2022, within all search fields in Scopus, there were 1737 document results, PubMed produced 228 results, Web of Science produced 91 results, and Google Scholar produced 3170 results. Because of the large amount of data retrieved from Scopus, the search was performed within the "Article title, Abstract, Keywords" field, and 771 publications were obtained. Further, Google Scholar and Scopus were used only for more refined searches. Those searches were mainly carried out for two sections: "Synthesis of testolactone" and "Biologically active derivatives and analogs of testolactone." The following terms were used for the search: "testolactone/synthesis," "testolactone/biotransformation," "testolactone/biological activity," "testolactone/derivatives," "testolactone/analogs," and "testolactone/modification." Analysis of the literature data led to the conclusion that testolactone is often confused or used interchangeably with testololactone [4,10–15]. From the structures of these two compounds presented in Figure 1, it can be observed that the correct synonym for testolactone is Δ^1-testololactone or 1-dehydrotestololactone. The papers that do not clearly state which compound is the subject of research are not covered by this review [16–18]. In addition, in the literature, it can also be found under the irregular compound name Δ^1-testolactone [19].

The fist synthesis of testolactone was published in 1953 by Fried et al. [20], while the first papers regarding biological activity were published by Segaloff et al. [21] and Lerner et al. [22] in 1960. Since then, the trend of publishing papers describing testolactone, synthesis, and biological properties has been changing and reached a peak at the end of the 1980s and the beginning of the 1990s, according to PubMed results (Figure 2a). According to Scopus, the peak was reached at the beginning of the 2000s (Figure 2b). Most of the published papers were research articles (67.5%), followed by reviews (23.4%) (Figure 3a). Only one of those reviews focused solely on testolactone [3]; the rest had various subjects, such as aromatase inhibitors, breast cancer, hormone therapy, etc. The main subject areas covered by the documents were medicine (52.9%), biochemistry, genetics and molecular biology (26.4%), pharmacology, toxicology and pharmaceutics (8.6%), and chemistry (4.5%) (Figure 3b).

Using VOSviewer software (v. 1.6.18, www.vosviewer.com, accessed on 18 November 2022), a map based on bibliographic data from PubMed was created, indicating connections between used keywords (Figure 4). The type of used analysis was the co-occurrence of keywords, while a minimum number of occurrences was set at five. Out of 787 keywords detected by PubMed, 113 met this threshold. The size of a keyword and bubble size indicates the number of publications in which the term was found, and the distance between two keywords offers an approximate relatedness. Furthermore, overlay visualization gave fast color-coded inside in connection between the keyword and year of publication (Figure 4). The most frequently repeated keywords in the works with testolactone are "human," "testosterone," and "aromatase inhibitors," while the newest are "child," "puberty, precocious," "antineoplastic agents," "infertility, male," "biotransformation," etc.

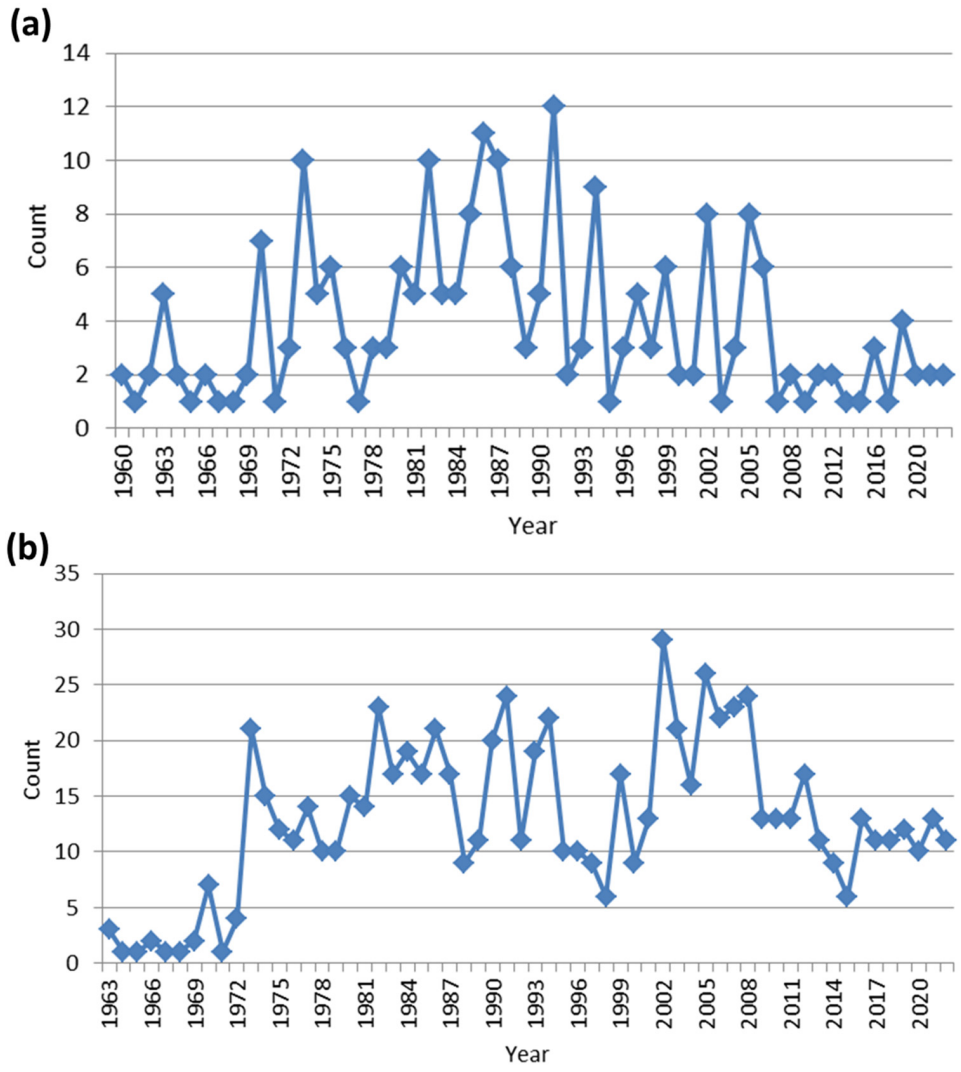


Figure 2. Number of published papers by year from (**a**) PubMed (results obtained using "testolactone" and the "All" search field), (**b**) Scopus (results obtained using "testolactone" and the "Article title, Abstract, Keywords" field).

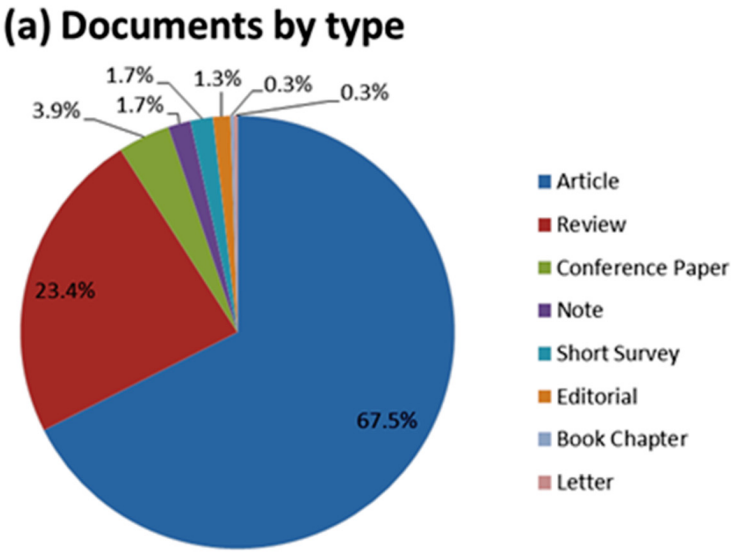


Figure 3. *Cont.*

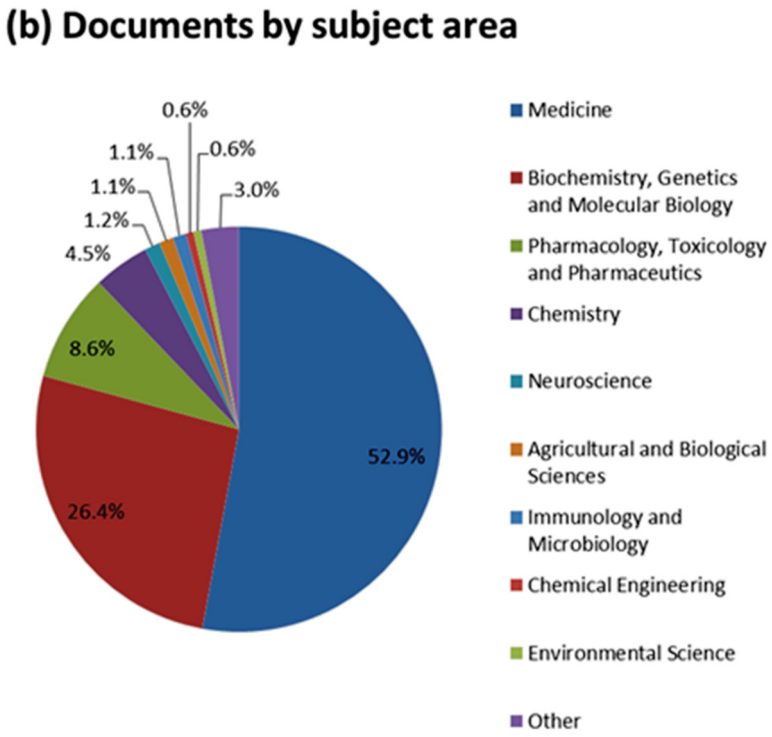


Figure 3. Distribution of documents by type (**a**) and by subject area (**b**) from Scopus (results obtained using "testolactone" and the "Article title, Abstract, Keywords" field).

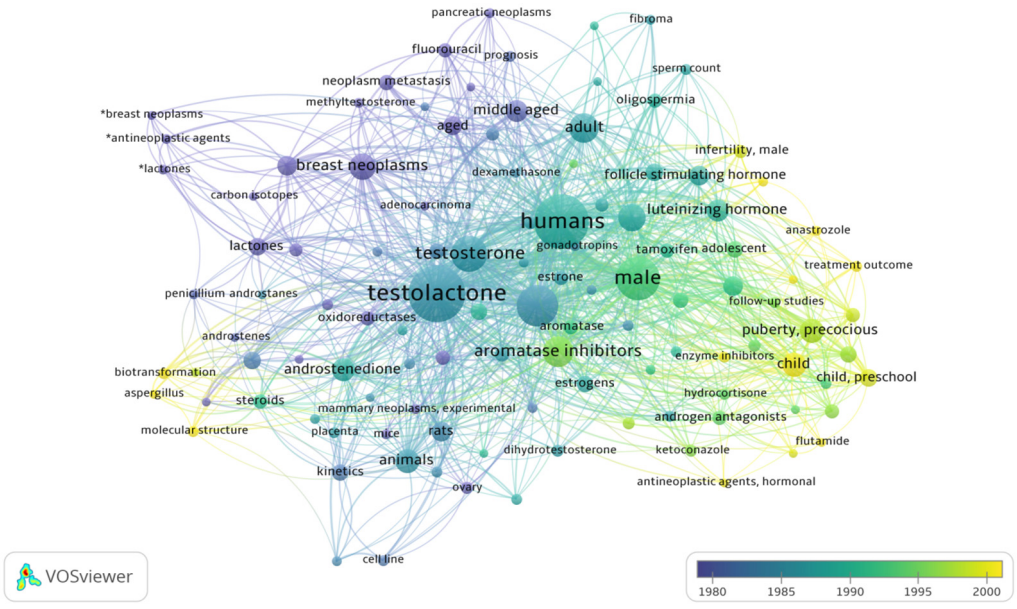


Figure 4. VOSviewer generated an overlay visualization graph with co-occurrence of keywords.

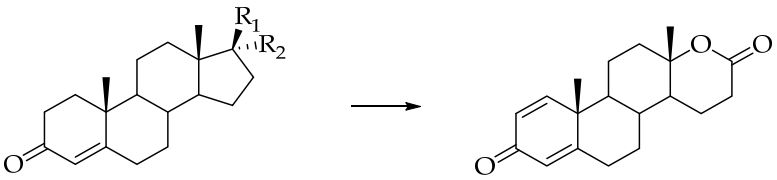
3. Synthesis of Testolactone

Pharmaceuticals with steroidal cores could be synthesized by classical chemical processes. However, microbial biotransformations of steroids, which have been known for several decades, are a good alternative, because their application offers many advantages over chemical synthesis: the high chemo-, regio-, and enantioselectivity, multiple successive reactions carried out in a single step, and more eco-friendly processes.

The chemical Baeyer–Villiger (BV) reaction transforms cyclic ketones into lactones by peracids, but this method is potentially dangerous because of the shock sensitivity and the

explosive character of peracids. These shortcomings of safety considerations and other limitations have been overcome by the biological BV reactions. The conversion of progesterone (**3**) to testolactone (**1**) or testololactone (**2**) by fungi, such as *Penicillium chrysogenum*, *Cylindrocarpon radicola*, or *Aspergillus flavus* provided the first evidence of lactone formation in the D-ring of steroids [20,23]. Baeyer–Villiger monooxygenases (BVMOs) are flavoenzymes that catalyze the Baeyer–Villiger reaction, thus converting ketones to corresponding esters or lactones, which are produced by numerous bacteria (e.g., of the genera *Actinetobacter Arthobacter*, *Nocardia*, *Rhodococcus*, *Streptomyces*) and fungi (e.g., of the genera *Aspergillus*, *Culvularia*, *Fusarium*, *Penicillium*) [24–26]. Many fungi can carry out the BV biotransformations of steroids [23,25,27–30], but bio-oxidation involving both lactone formation in ring D and dehydrogenation in ring A is less widespread and has been observed with a small number of organisms [20,31,32].

Fried et al. [20] described the conversion of the 21-unsubstituted 3,20-diketopregn-4-ene and androstane series to the testolactone series by microorganisms of the family *Tuberculariaceae*. When progesterone (**3**), Reichstein's compound S (**4**), or testosterone (**5**) were fermented by *Cylindrocarpon radicola*, testolactone (**1**) was obtained in about 50% yield (Scheme 1) [20,31].

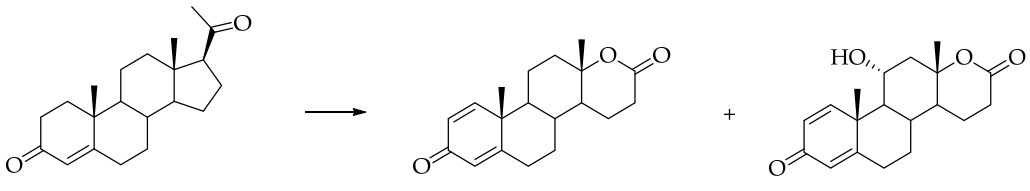


3, R_1=$COCH_3$, R_2=H
4, R_1=$COCH_2OH$, R_2=OH
5, R_1=OH, R_2=H

1

Scheme 1. Transformation of steroids by *Cylindrocarpon radicola*.

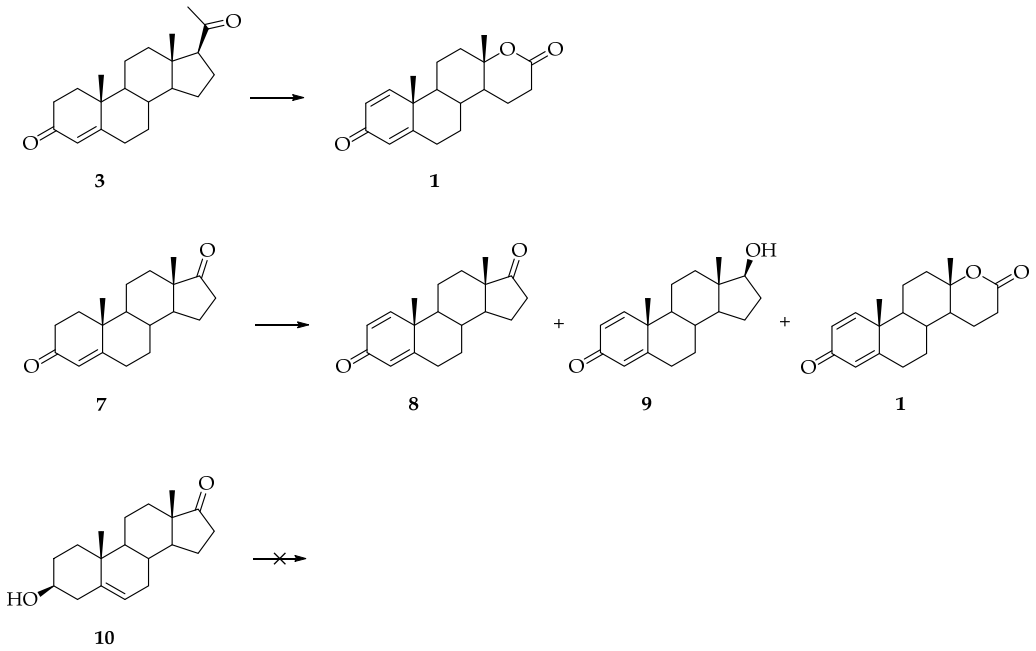
Subjecting progesterone (**3**) to the action of the enzymes of fungi of the genus Fusarium (*Fusarium javanicum* var. *ensiforme*), testolactone (**1**) was obtained as the major product and 11α-hydroxytestolactone (**6**) as a byproduct (Scheme 2) [31].



3 **1** **6**

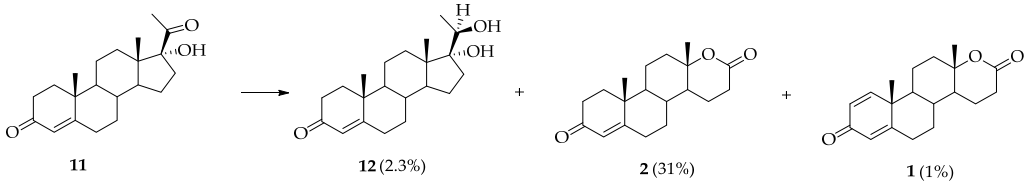
Scheme 2. Transformation of steroids by *Fusarium javanicum* var. *ensiforme*.

Čapek et al. [33] subjected progesterone (**3**), androst-4-ene-3,17-dione (**7**), and dehydroepiandrosterone (**8**) to microbial transformation with *Fusarium lateritium* (Scheme 3). When progesterone (**3**) was used as starting compound, testolactone (**1**) was obtained with around 40% yield. Androst-4-ene-3,17-dione (**7**) gave a mixture of androsta-1,4-diene-3,17-dione (**8**), and 1-dehydrotestosterone (**9**) in a ratio of 9:1, while testolactone (**1**) was formed in traces (about 2%). Additionally, dehydroepiandrosterone (**10**) was not transformed by the *Fusarium lateritium*.



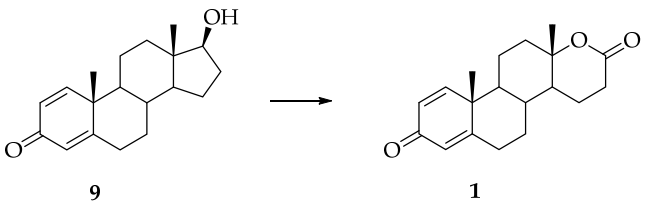
Scheme 3. Transformation of steroids by *Fusarium lateritium*.

Hunter et al. [34] carried out the transformation of a range of cortical steroids by the fungus *Aspergillus tamarii*, which can convert progesterone (**3**) to testololactone (**2**) in high yield. Incubation of 17α-hydroxyprogesterone (**11**) resulted in three products: 17α,20(*R*)-dihydroxypregn-4-en-3-one (**12**), testololactone (**2**), and testolactone (**1**) in trace (Scheme 4).



Scheme 4. Transformation of 17α-hydroxyprogesterone (**11**) by *Aspergillus tamarii*.

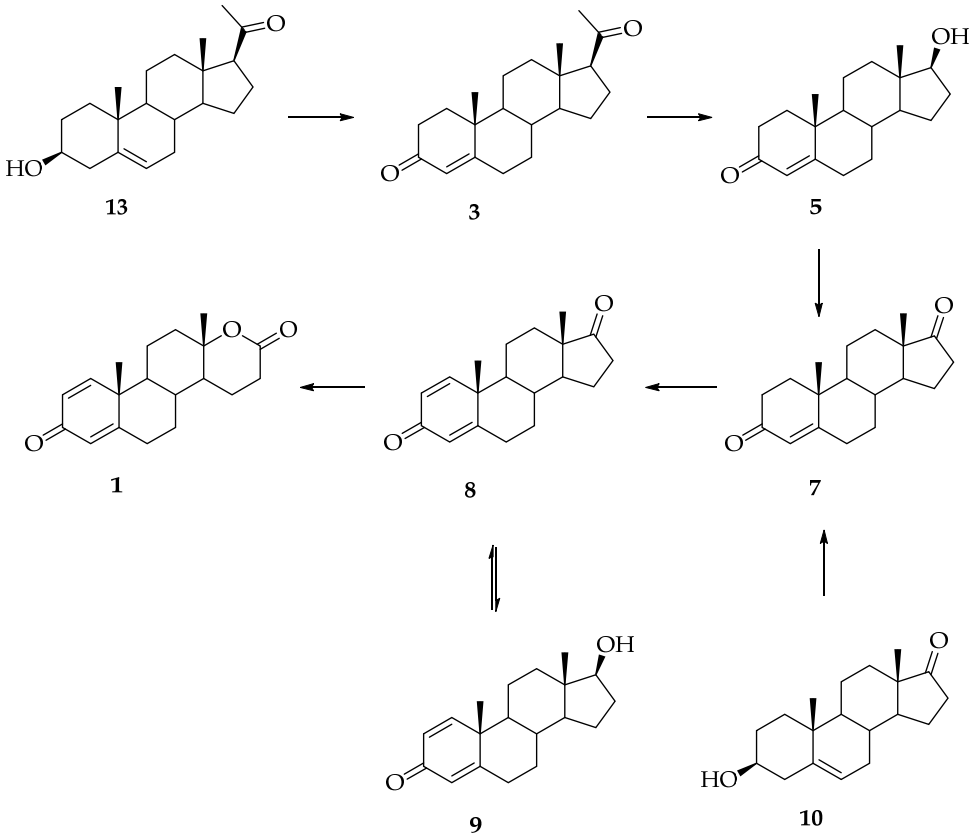
Furthermore, Bartmanska et al. [13] investigated the application of *Penicillium notatum* for biotransformations of steroids. The fungus can perform an oxygen atom insertion into the D-ring and when 1-dehydrotestosterone (**9**) was used as starting compound (Scheme 5), testolactone (**1**) was the only product isolated from the chloroform extracts in the yield of 90%.



Scheme 5. Transformation of 1-dehydrotestosterone (**9**) by *Penicillium notatum*.

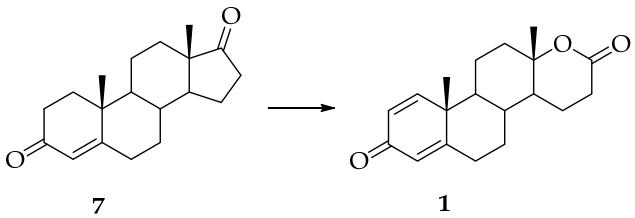
Testolactone was synthesized from dehydroepiandrosterone (**10**) using *Fusarium oxysporum* SC1301 isolated from soil samples, which, in addition to dehydroepiandrosterone (**10**), also transformed androst-4-ene-3,17-dione (**7**), androst-1,4-diene-3,17-dione (**8**), progesterone (**3**), testosterone (**5**), and pregnenolone (**13**) to testolactone (**1**), in yields of 76–98% (Scheme 6). This indicates the ability of *Fusarium oxysporum* SC1301 to enable multistep functional transformations, including oxygenative esterification of 20-ketosteroids, hydrol-

ysis of esters, oxidation of C-17 OH groups, oxygenative lactonization of 17-ketosteroids, 1-dehydrogenation, oxidation of C-3 OH groups and $\Delta^{5\rightarrow4}$ isomerization in an efficient way to obtain one product [35].



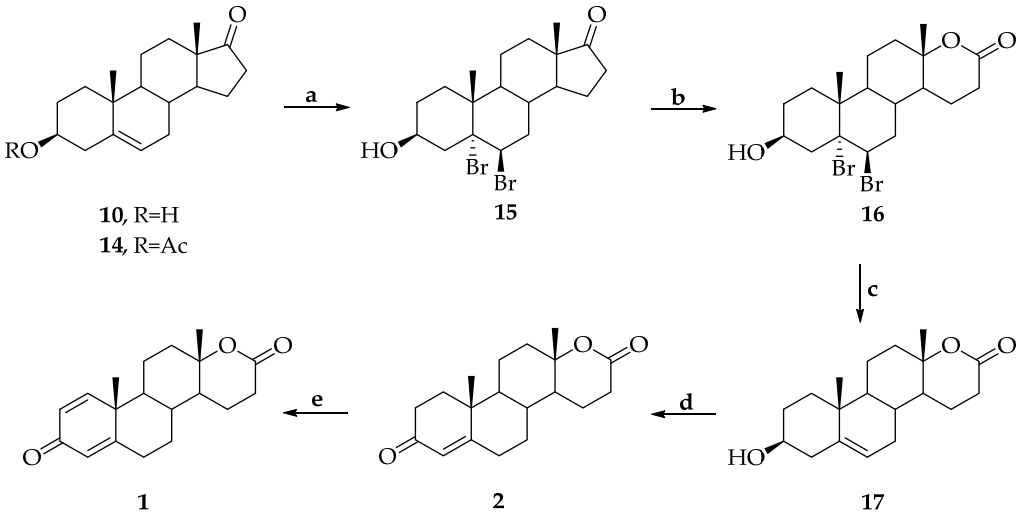
Scheme 6. Transformation of steroids **3**, **5**, **7**, **8, 10**, and **13** to testolactone (**1**) by *Fusarium oxysporum* SC1301.

On the other hand, direct bioconversion of androst-4-en-3,17-dione (**7**) with the simultaneous dehydrogenation and oxidation to testolactone (**1**) in high yield was achieved by submerged culture of *Fusarium solani* ATCC46829 (Scheme 7) [32].



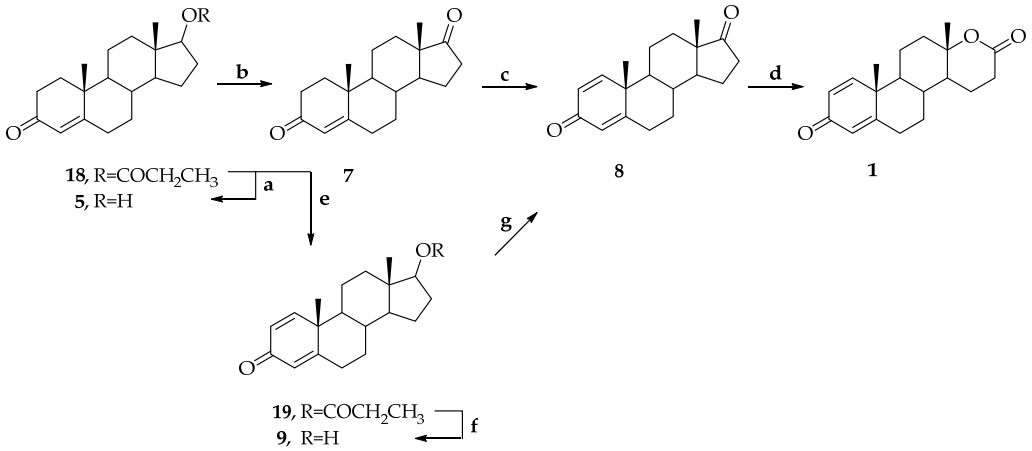
Scheme 7. Transformation of androst-4-en-3,17-dione (**7**) by *Fusarium solani* ATCC46829.

Although, microbial production is much more environmentally friendly, testolactone (**1**) has also been synthesized by a chemical route. The first conventional synthesis of testolactone (**1**) from dehydroepiandrosterone (**10**) is shown in Scheme 8. The strategy in this synthetic approach included the introduction of the dienone system after the formation of the D-lactone ring. Upon protection of a double bond of compound **10** or **14** in form of the dibromide, followed by Baeyer–Villiger oxidation of **15** with peracetic acid, the D-lactone ring was formed (compound **16**). After Oppenauer oxidation of deprotected derivative **17**, a double bond between C1 and C2 in ring A of compound **2** was introduced by oxidation with selenium dioxide [36].



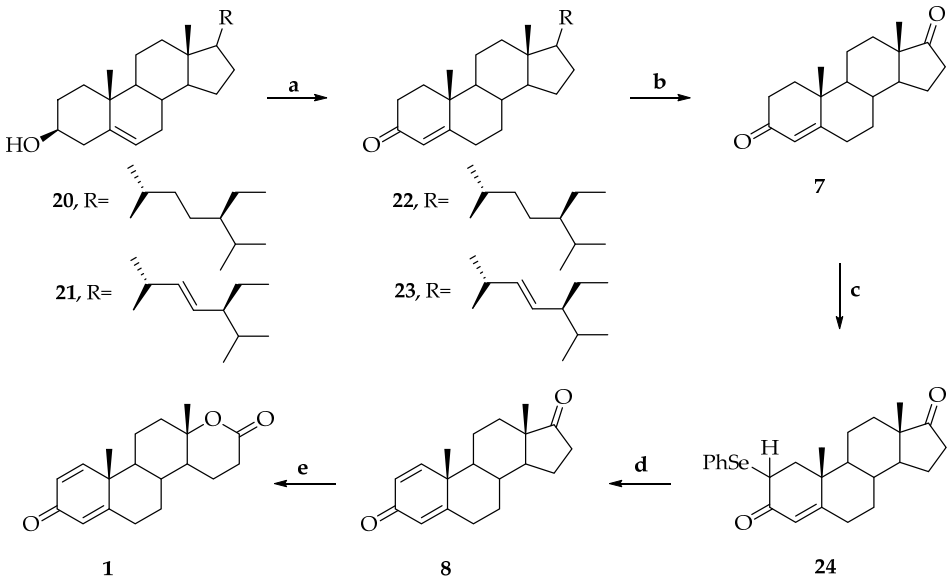
Scheme 8. Chemical transformation of dehydroepiandrosterone (**10**) to testolactone (**1**). Reagent: (**a**) bromine; (**b**) peracetic acid; (**c**) sodium iodide; (**d**) Oppenauer oxidation; (**e**) selenium dioxide.

Zinczuk et al. [37] have modified the classical synthesis of testolactone (**1**). The strategy in their synthetic approach consisted in the formation of the D-lactone ring upon generation of the dienone system. Testosterone propionate (**18**) was used as starting compound for the preparation of dienone **8** (Scheme 9). Upon saponification of **18**, followed by oxidation of **5** with pyridinium chlorochromate (PCC), androst-4-ene-3,17-dione (**7**) was obtained in good yield. For the introduction of the 1,4-diene system into the A-ring of compound **7**, 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) was used and compound **8** was obtained. Compound **8** can be also prepared from compound **18** in treatment with DDQ in the presence of benzoic acid in toluene, when compound **19** was obtained. Further saponification of compound **19** resulted in compound **9**, which, after oxidation with Jones reagent, gave compound **8**. In a different reaction pathway, upon oxidation of compound **18** with nontoxic 2-iodoxybenzoic acid (IBX), and saponification of **19,** followed by oxidation of **9** with IBX, compound **8** was also obtained in good yield. For the formation of the D-lactone ring, explosive peracetic acid was replaced with the safer and inexpensive monoperoxyphthalic acid magnesium salt hexahydrate (MMPP), and testolactone (**1**) was obtained from **8** in the yield of 98%. By using reagents such as IBX and MMPP, an efficient and environmentally benign chemical synthesis of testolactone (**1**) was conducted.



Scheme 9. Chemical transformation of testosterone propionate (**16**) to testolactone (**1**). Reagent and reaction condition: (**a**) KOH, H_2O, EtOH, reflux; (**b**) PCC, SiO_2, CH_2Cl_2, rt; (**c**) DDQ, PhCOOH, $PhCH_3$, reflux; (**d**) MMPP, AcOH, rt; (**e**) DDQ, $PhCH_3$, 90 °C or IBX, DMSO, 85 °C; (**f**) K_2CO_3, MeOH, rt; (**g**) Jones reagent or IBX, DMSO, rt.

Testolactone (**1**) was also synthesized using the naturally occurring β-sitosterol (**20**) and stigmasterol (**21**) as starting compounds (Scheme 10). This synthetic pathway involved Oppanauer oxidation of both β-sitosterol (**20**) and stigmasterol (**21**) to generate an enone system in A ring. Compound **22** or **23** was then subjected to oxidative cleavage of the side chain to produce androst-4-ene-3,17-dione (**7**). Dehydrogenation of compound **7** with phenylselenyl chloride in ethyl acetate, followed by selenium oxide elimination with H_2O_2 in dichloromethane, provided compound **8**. Baeyer–Villiger oxidation of **8** yielded testolactone (**1**) with overall yield of 33%. This procedure may be exploited for the bulk production of Teslac from the most abundant and naturally occurring phytosterols such as β-sitosterol (**20**) [38].



Scheme 10. Hemisynthesis of testolactone (**1**) from β-sitosterol (**20**) and stigmasterol (**21**). Reagent and reaction condition: (**a**) Al(*i*-PrO)$_3$, ciklohexanone, toluene, reflux; (**b**) *Micobacterium sps* (NRRLB-3805); (**c**) PhSeCI, EtOAc, rt; (**d**) H_2O_2, DCM; (**e**) *m*-CPBA, DCM.

4. Medicinal Uses, Benefits and Side Effects of Testolactone

When testolactone became available in the 1960s of the last century, and especially after preliminary biological tests, there was an expansion of works focusing on biological activity and the clinical application of testolactone. Testolactone was approved for clinical use in 1969 by the Food and Drug Administration (FDA), and, in 1970, testolactone appeared under the trade name Teslac in the production of E. R. Squibb and Sons, while official literature on new drugs was published in Clinical Pharmacology and Therapeutics [39]. The precise mechanism by which testolactone exerts its clinical antineoplastic effects was still unknown. Assumptions about mechanism of action were diverse, and it was definitely determined that it is an inhibitor of the enzyme aromatase, which participates in the conversion of androstenedione into estrone, and testosterone into estradiol [2]. In addition, antiandrogenic properties and binding to androgen receptors were investigated, and even controversial research was published [19,22]. Interestingly, many clinical trials in breast carcinoma patients were performed before its mechanism of action was known. Unfortunately, weak clinical activity led to its withdrawal from the market. While its application was most often based on the inhibition of the enzyme aromatase, now, the mechanism of action and the influence on the production and circulation of sex hormones are well known. Aromatase is a cytochrome P450 (CYP19) enzyme that is involved in the conversion of androgens to estrogens. In higher concentration, aromatase is expressed in breast cancer and plays a pivotal role in the origin and progression of breast cancer [40–42].

In order to study the mechanism of aromatization of androgens into estrogens in biological systems, Siiteri et al. [2] carried out studies of human placental aromatase, where they hypothesized that antitumor activity of steroidal drugs might be due to the inhibition of estrogen synthesis, rather than direct androgen action. Authors have concluded that the aromatase-inhibiting action of testolactone has been attributed to its competition with testosterone and androstenedione for microsomal aromatase cytochrome P450 [2], leading to a decrease in estrogen synthesis.

In further research, Covey and Hood [43] examined five aromatase inhibitors (4-hydroxyandrost-4-ene-3,17-dione; androst-4-ene-3,6,17-trione; androsta-1,4,6-triene-3,17-dione; androsta-1,4-diene-3,17-dione; and testolactone) to explain the unexpected loss of aromatase activity caused by the tested inhibitors. They have concluded that the tested aromatase inhibitors redirect the third oxidation step with the covalent bonding of the steroid to the enzyme, which results in irreversible enzyme inactivation, while the 3-keto-4-ene system, also present in testolactone, displays a kinetic profile expected for a suicide substrate. This assumption about the irreversible inhibition of aromatase was further studied by Klein et al. [44] in their research on the perfusion of the human placenta, and the results were compared with those from kinetic experiments in a cell-free system. They have concluded that examined inhibitors (1-methylandrost-1,4-diene-3,17-dione; 19-azidoandrost-4-ene-3,17-dione; 4-hydroxyandrost-4-ene-3,17-dione; and testolactone) caused irreversible aromatase inactivation in a cell-free system, but this phenomenon could not be observed during the perfusion experiments.

In their work, Lerner et al. [22] investigated the biological properties of testolactone in several species of laboratory animals. They have concluded that testolactone is not androgenic, estrogenic, progestational, gonadotropin-like, anti-progestational, antigonadotropic, or antiestrogenic in nontoxic doses, and has no biological effect on androgen-dependent organs. Although this study suggested that testolactone has no intrinsic estrogenic or androgenic activity, Vigersky et al. [19] demonstrated that testolactone has antiandrogenic properties in addition to its aromatase-inhibiting activity, because it competitively interacts with dihydrotestosterone for the androgen receptors. Possible reasons for these contradictory results were in the administered doses and the length of therapy used in earlier works; Vigersky et al. [19] concluded that the antiandrogenic activity of testolactone in rats in vivo is dose and time-dependent. Finally, the authors concluded that testolactone, as an antiandrogen, could have advantages over other antiandrogens, because it inhibits the conversion of testosterone to estradiol, reduces the effective ratio of testosterone to estradiol, and, as a result, estrogenic side effects, such as gynecomastia, may be less frequent in men on therapy. Additionally, to determine the relative potencies of antiandrogenic compounds in human cells, Eil and Edelson [45] measured the abilities of various compounds to compete with dihydrotestosterone for androgen-binding sites in dispersed human genital skin fibroblasts, but testolactone was one of the weakest antiandrogen among the tested substances (spironolactone > trimethyltrienolone > megestrol-acetate > cyproterone-acetate > estradiol > flutamide >> testolactone > cimetidine).

4.1. Cancer

4.1.1. Breast Cancer

One of the first papers on the use of testolactone for the treatment of breast cancer was published in 1960 by Segaloff et al. [21]. In their research, they observed sustained objective remission of the advancing breast cancer. In addition to the effectiveness of parenteral therapy with testolactone, later in their work, they investigated the efficacy of the oral administration of this agent, where testolactone caused a higher response rate in oophorectomy failures than other hormonal agents, with objective regression of disease, and increase in excretion on 17-ketosteroids, and no evidence of androgenicity [46]. Further investigations were conducted by Cantino et al. [47] when they increased the dose of testolactone in an attempt to obtain better control of the disease, but without improvement in the objective regression rate.

In order to develop potent anticancer agents, The National Cancer Institute has been working with the Cooperative Breast Cancer Group since 1957. For that reason, Goldenberg performed clinical studies in which breast cancer was treated with testolactone, medroxyprogesterone acetate, or oxylone acetate, where testolactone produced the lowest objective remissions, while the observed survival experience was the best for the patients receiving testolactone and poorest for those receiving medroxyprogesterone acetate [48]. In further study of advanced breast cancer in women, Goldenberg et al. [49] applied testolactone as a reference substance to confirm the biological activity of calusterone. In their research, a randomized clinical trial of calusterone and testolactone produced objective remissions of advanced breast cancer in 28% of women receiving calusterone and 18% of those given testolactone. In the next report of the Cooperative Breast Cancer Group, Goldenberg et al. [50] described a clinical trial of aromatase inhibitor and antimetabolite therapy for advanced female breast cancer, where an objective regression rate of 20% was achieved in women receiving oral testolactone, 6% in patients given intravenous fluorouracil alone, and 14% when they were administered together.

Barone et al. [51] examined postmenopausal women with metastatic breast cancer on oral therapy with testolactone. Based on measuring the levels of estrone, serum androstenedione, and testosterone, they came to the conclusion that testolactone is a potent inhibitor of peripheral aromatization of androstenedione into estrone, without affecting serum androstenedione and testosterone levels. In further in vitro studies it was determined that estrogen formation can be blocked directly at the tumor site and in addition to reducing of the level of plasma estrone, estradiol was lowered [52]; moreover, testolactone can block peripheral formation of estrogens in breast tumors [53]. Further, Kaufman [54] analyzed the impact of estrogen, progestin, androgens, corticosteroids, and testolactone in combination with non-hormonal therapy for the treatment of breast cancer, and pointed out that additive hormonal therapy still has a major role in the management of the advanced breast cancer patient.

4.1.2. Prostatic Carcinoma

The treatment of hormone-dependent prostate cancer, aiming at reducing circulating androgen levels, mainly estrogen administration, gained popularity in the 1980s of the last century. The mechanisms behind the beneficial effects of estrogens on prostatic carcinoma were not yet known. In order to investigate the role of estrogens in human male endocrinology Leinonen et al. [55] performed a study where tamoxifen or testolactone was given to prostatic carcinoma patients, concluding that in vivo contribution by testolactone or its metabolites was very small.

4.1.3. Human Benign Prostatic Hyperplasia (BPH)

Testolactone was applied for endocrine treatment of benign prostatic hyperplasia (BPH), since there was an assumption that estrogens might also be causally related to the onset and maintenance of BPH. This approach was defined by Schweikert and Tunn [56], because at that time it was known that, among other, peripheral aromatization of circulating androgens is the major source of estrogen in men, and the amount of aromatized androgens increases with advancing age. While, aromatization of androstenedione to estrone has the highest conversion rates in cells derived from BPH tissue, authors have used testolactone for endocrine treatment of benign prostatic hyperplasia (BPH) in thirteen patients. As a result, prostatic volume decreased.

In further studies of BPH, Bartsch et al. [57] investigated the presence of aromatase in benign prostatic hyperplasia and possible inhibition of the enzyme 17β-hydroxysteroid dehydrogenase (17βHSDH). Bartsch et al. [57] have investigated whether aromatase inhibiting compounds might partially act by inhibition of 17βHSDH, but beneficial effects of testolactone observed in BPH patients were due to inhibition of aromatase and not of 17βHSDH.

4.1.4. Desmoid Tumors

The use of testolactone in the treatment of desmoid tumors is also described in the literature. Considering that there was an assumption that these tumors were hormone dependent, and additionally, estrogen receptors have been found in 33 to 75% of the desmoid tumors, the idea of using aromatase inhibitors in their treatment arose [58]. Research by Waddell [59] concerns the chemical treatment of desmoid tumors with testolactone and subsequently with theophylline and chlorothiazide. These compounds are inhibitors of cyclic adenosine monophosphate (cAMP) phosphodiesterase (PDE), which is known to have in vitro effects on tumor cells, while testolactone by itself has some inhibitory effect on cAMP phosphodiesterase. The authors have concluded that the combination of these three drugs synergize the action of 3′,5′-adenosine monophosphate and the latter two may work by inhibiting the action of 3′,5′-adenosine monophosphate diesterase. Waddell and Kirsch [60] in their further research have investigated combination therapy regimens consisting of steroidal aromatase inhibitor (testolactone) and nonsteroidal anti-inflammatory drugs (indomethacin, sulindac, warfarin and vitamin K1). Their therapeutic recommendations minimized the effects of estrogen and inhibited prostaglandin and cAMP synthesis, which have influence on growth and proliferation of fibroblasts. Furthermore, Gansar and Krementz [61] published a case of a patient with a desmoid tumor, which was treated with testolactone and chlorothiazide, where a marked decrease in pain was reported, along with a change in the shape of the lesions.

Tsukada et al. [58] investigated noncytotoxic drug therapy for the treatment of intra-abdominal desmoid tumors in patients with familial adenomatous polyposis (FAP). They have concluded that for female patients, tamoxifen may be used, and in male patients intolerant of NSAIDs, progesterone or testolactone may have some effect.

4.1.5. Carcinoma of the Pancreas

The results of two chemotherapy regimens for inoperable carcinoma of the pancreas have been described. One group was treated with 5-fluorouracil and coumadin, while the other was treated with 5-fluorouracil and testolactone or spironolactone. The authors have concluded that testolactone has no significant additive benefits in prolonging survival in combination treatment programs [62–64].

4.2. Precocious Puberty

If a boy develops secondary sexual characteristics before the age of 9 years or a girl before the age of 8 years, the child is considered to have sexual precocity. They can be classified into variations of normal pubertal development, and contrasexual pubertal development: central precocious puberty (CPP), which results from early activation of the hypothalamic–pituitary–gonadal axis; or peripheral precocious puberty (PPP), which appears due to extrapituitary gonadotrophin secretion or independent sex steroid secretion [65,66]. Further, familial male-limited precocious puberty (FMPP), is caused by a mutation in the luteinizing hormone/chorionic gonadotropin receptor gene, resulting in the receptor being constitutively activated, which causes excessive production of testosterone [67]. In order to mitigate the effects of estrogen on growth, skeletal maturation, and secondary sexual development, aromatase inhibitors began to be used in the mid-1980s. In the treatment of selective forms of precocious puberty, testolactone has been demonstrated to be tolerable and effective, while diverse results with testolactone have been achieved in girls with McCune–Albright syndrome [68].

In their research, Laue et al. [69] hypothesized that the blockade of androgen action and estrogen synthesis would normalize the growth of boys with familial male-limited precocious puberty (FMPP) because puberty in boys appears to be mediated by both androgens and estrogens. To test this hypothesis, authors studied nine boys during treatment with an antiandrogen (spironolactone) or an inhibitor of aromatase (testolactone), followed by treatment with both agents. Only a combination of spironolactone and testolactone, given for at least six months, restored both the growth rate and the rate of bone maturation

to normal prepubertal levels and controlled acne, spontaneous erections, and aggressive behavior. Blockade of both androgen action and estrogen synthesis is an effective short-term treatment for familial male precocious puberty. While FMPP is a luteinizing-hormone-releasing hormone-independent (LHRH-independent) form of precocious puberty, in the continuation of their research, Laue et al. [70] have included testolactone and spironolactone in the therapy with LHRH agonist deslorelin, which have been effective as single agents in the treatment of LHRH-dependent precocious puberty. Combined therapy decreased peak LH, plasma testosterone, bone maturation rate, and growth velocity. They have concluded that the rise in gonadotropin levels during central activation of hypothalamic LHRH secretion in boys with familial male-limited precocious causes a partial escape from the combined effect of spironolactone and testolactone. The addition of deslorelin to the combined therapy appears to restore the control of puberty in this setting.

In their work Cummings et al. [71] examined whether elevations in testosterone and androstenedione recorded during treatment in FMPP were caused by interference of testolactone and spironolactone in radioimmunoassays. They measured serum levels of testosterone and androstenedione serially by RIA for 24 h in a girl with McCune–Albright syndrome, a boy with familial male precocious puberty, and in a healthy postmenopausal control after a single oral dose of testolactone. They have concluded that testolactone significantly interferes in these serum RIAs, making their use unreliable in the follow-up of patients treated with testolactone. On the other hand, Werber Leschek et al. [72] confirmed the research findings of Laue et al. [69,70] in boys with FMPP, and further, they evaluated the effect of long-term antiandrogen (spironolactone), an aromatase inhibitor (testolactone or anastrozole), and gonadotropin-releasing hormone analog (GnRHa; deslorelin or depot leuprolide) on adult height in boys with FMPP. In their thirty-year study, testolactone was replaced by anastrozole after it was withdrawn from the market. The combined regimen of an antiandrogen, an aromatase inhibitor, and GnRHa substantially achieved the study goals of normalizing growth, development, and adult height [73].

Precocious puberty in girls with McCune–Albright syndrome (MAS) is usually due to ovarian estrogen secretion that is independent of pubertal hypothalamic pituitary activation. Isosexual precocity, due to increased ovarian estrogen secretion with recurrent formation of ovarian cysts, is the most endocrine abnormality in girls with McCune–Albright syndrome. The lack of an effect of LHRH agonists on the clinical and biochemical sings, suggests that sexual precocity is maintained by gonadotropin-independet, autonomous ovarian estrogen secretion. Because, treating with medroxyprogesterone and cyproterone acetate was not successful in suppressing all sings, Foster et al. [74] have treated a girl with McCune–Albright syndrome with testolactone. During testolactone therapy, menses ceased, bone age advancement and height velocity diminished, and plasma estradiol levels were suppressed. Serum gonadotrophin levels remained in the prepubertal range and testolactone was an effective therapy of precocious puberty in girls with McCune–Albright syndrome. Further, Hauffa et al. [75] have compared the short-term effects of testolactone, with those of cyproterone acetate, a combined therapy with medroxyprogesterone and spironolactone, and of surgery alone. In their conclusion, therapy with testolactone led to an absolute and relative decrease in height velocity, a normal rate of bone maturation, and to elevation of androstenedione plasma concentrations.

In their research of precocious puberty in girls with McCune–Albright syndrome, Feuillan et al. [76] concluded that testolactone decreased the levels of circulating estradiol and the ovarian volume, leading to a return to pretreatment levels after testolactone was stopped. Further, Feuillan et al. [77] applied testolactone therapy to two girls who did not have skin and bone lesions, but who developed precocious puberty independent of luteinizing hormone and were unresponsive to LHRH agonist therapy. One of these girls appeared to have benefited from testolactone. In their further studies of long-term use of testolactone therapy, they concluded that testolactone can be effective in the treatment of LHRH-independent precocious puberty in girls with McCune–Albright syndrome, but some patients exhibit an escape from the effects of the treatment after 1–3 years [78].

Gryngarten et al. [79] analyzed the cases of girls who were treated at the Department of Endocrinology of Hospital de Niños Ricardo Gutiérrez between 1974 and 2019. In their work they have applied medroxyprogesterone acetate, tamoxifen, testolactone, or letrozole in the treatment of these girls and have concluded that these drugs can contribute to the treatment of certain aspects of this illness, they can improve the quality of life.

Even though, MAS is predominantly observed in girls and is rarely reported in males, Papadopoulou et al. [80] report the case of a 9-year-old boy with gonadotropin-independent precocious puberty, café-au-lait spots, polyostotic fibrous dysplasia and growth hormone hypersecretion. After diagnosis, treatment with somatostatin long-acting analogs and testolactone was initiated. Testolactone was discontinued at the age of 13 and central puberty began shortly thereafter.

In her work, Zacharin [81] described the case of a boy with polyostotic fibrous dysplasia, acromegaly, and gonadotrophin-independent precocious puberty, which was treated with flutamide in combination with testolactone. An increase in hepatic enzymes and neutropaenia during the first 3 months was thought to be due to flutamide. Withdrawal of flutamide resulted in rapid normalization of the white cell count and reduction in liver enzymes. Testolactone was continued without further problems, which lead to a significant improvement in quality of life.

4.3. Gynecomastia

Gynecomastia is a condition of non-cancerous overdevelopment or enlargement of the breast tissue in men or boys. An imbalance of estrogen and testosterone in the body often leads to this condition, while in most cases, the tissue disappears spontaneously within a few years, but in some cases, hormone therapy was applied.

In their work Zachman et al. [82] described the use of testolactone in the treatment of boys with pubertal gynecomastia, where a significant increase in androstenedione levels and a significant decrease in mean breast diameter were observed. Similarly, Binder et al. [83] studied a family in which seven affected males over three generations had inherited prepubertal gynecomastia in an autosomal dominant manner. In their work, treatment was more effective with anastrozole than with testolactone and increased the initially reduced testes volume to normal size, promoted virilization, and normalized serum estrone and testosterone levels.

In the case report presented by Auchus and Lynch [84], testolactone was administered to men with unilateral gynecomastia, occurring after unilateral orchiectomy. Testolactone effectively treated this condition by raising the androgen-estrogen ratio without lowering absolute estradiol levels.

Kara et al. [85] studied a case of a boy with Peutz–Jeghers syndrome, bilateral gynecomastia, Sertoli cell tumor, and nephrocalcinosis. The aromatase inhibitor testolactone was administered in an attempt to prevent skeletal maturation. One-year treatment with testolactone reduced the breast base diameter, and there was no rapid advancement in his bone age.

4.4. Congenital Adrenal Hyperplasia (CAH)

Congenital adrenal hyperplasia (CAH) is an autosomal recessive condition with reduced glucocorticoid and mineralocorticoid biosynthesis, as a result of deletions or mutations of the cytochrome P450 21-hydroxylase gene, which leads to increased secretion of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), adrenal hyperplasia, and excessive production of cortisol and aldosterone [86,87].

Merke et al. hypothesized that by using antiandrogens in combination with an inhibitor of estrogen synthesis from androgens, the hydrocortisone dose could be reduced [87–90]. They have investigated the impact of the regimen of antiandrogen (flutamide), an aromatase inhibitor (testolactone), reduced hydrocortisone dose, and fludrocortisone compared to the effects of a control regimen of hydrocortisone and fludrocortisone in children with classic 21-hydroxylase deficiency. Additionally, they investigated the effect of flutamide

alone, on cortisol clearance. Their findings demonstrate that the addition of flutamide and testolactone to the treatment regimen of hydrocortisone, significantly decreases cortisol clearance, but this effect seems to be due to flutamide [87].

4.5. Fertility and Sterility

In the 1980s, one of the research directions on fertility and sterility was based on the assumption that estradiol can indirectly affect spermatogenesis by preventing the Leydig cell from maximally producing testosterone in response to the luteinizing hormone. It was considered that, by decreasing estradiol formation, there might be an improvement in both sperm count and fertility in patients with infertility due to oligospermia. In this respect, Vigersky and Glass [91] investigated the influence of testolactone on spermatogenesis and the pituitary–testicular axis in men with idiopathic oligospermia. They concluded that after testolactone therapy there was a decrease in estrone and estradiol levels, followed by an increase in serum testosterone and androstenedione; further, there was an increase in the testosterone-estradiol ratio, and thus an improvement in spermatogenesis, with no effect on gonadotropin production in men.

In their works, Dony et al. [92,93] investigated whether sustained lowering of endogenous estrogen levels by chronic administration of testolactone might influence the anomalous Leydig cell response to luteinizing hormone-releasing hormone in patients with idiopathic oligospermia. They have concluded that testolactone lowered circulating estradiol, and thereby sex hormone binding globulin (SHBG), enhanced the secretion of follicle-stimulating hormone (in contrast to the investigation of Vigersky and Glass [91]), 17-hydroxyprogesterone and testosterone, but did not affect serum luteinizing hormone levels. Despite estradiol lowering, there was an accumulation of 17-hydroxyprogesterone over testosterone, suggesting 17,20-lyase inhibition. Although these investigations suggest that estrogens play a less dominant role in the origin of the late steroidogenic lesion than previously assumed, the suggestion also arises that testolactone per se, in addition to its antiestrogenic action, has an inhibiting effect on the 17,20-lyase, which may obscure the beneficial effect of reducing estradiol.

Maier and Hienert [94] investigated whether inhibition of testosterone catabolism by testolactone prevents the increase in the estradiol concentration during tamoxifen therapy and whether the ejaculate parameters or the incidence of gravidity can be further improved, compared to monotherapy with tamoxifen. Their results indicate that with additional administration of testolactone, the increase in estradiol levels on tamoxifen therapy is reduced, but not completely eliminated. With a combination therapy of tamoxifen and testolactone, no further improvement of the ejaculate parameters was seen and there is no advantage over monotherapy with testolactone.

Pavlovich et al. [95] identified an endocrinopathy in men with severe male factor infertility, characterized by a decreased serum testosterone-to-estradiol ratio. This ratio can be corrected when men were treated with testolactone, resulting in a significant improvement in semen parameters in oligospermic patients.

Dunaif et al. [96] have investigated the effects of testolactone on gonadotropin release and steroid metabolism in polycystic ovarian disease (PCOD). In their conclusions, testolactone was potent inhibitor of peripheral, but not ovarian aromatase in humans, and of hypothalamic aromatase in rats.

In order to obtain additional information concerning the site of action of testolactone on human testicular steroid production, Martikainen et al. [97] studied the effects of testolactone on a number of circulating steroid hormones and their sulfate conjugates and on serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), and sex-hormone-binding globulin (SHBG) concentrations. Short-term testolactone treatment caused a partial inhibition of aromatization of androgens to estrogen in normal men and further inhibitory influence on the 17β-reduction of dehydroepiandrosterone and androstenendione. Increased serum concentrations of pregnenolone, 17-hydroxypregnenolone, and

testosterone sulfates were also observed, but it remains unclear whether testolactone has a direct or possibly an estradiol-mediated effect on the steroid sulfates of testicular.

In order to assess the hormonal alterations that occur in the testolactone-therapy, Nagler et al. [98] used adult male rat model for studying the role of estrogens upon sperm production. They concluded that testolactone does not have an effect on the peripheral level of testosterone or estradiol and the data suggests that the blood production rate of estradiol in the male rat is not dependent upon the aromatization of peripheral testosterone.

Testolactone was used in order to examine whether there is feedback regulation of androgens/estrogens on prolactin secretion in the human male, while there is evidence that prolactin may be involved in testicular steroidogenesis. Gooren et al. [99] have measured basal and thyrotropin-releasing hormone (TRH)-stimulated prolactin levels in eugonadal and agonadal men after testolactone, spironolactone or dihydrotestosterone undecanoate treatment. They have concluded that in the human male, endogenous estrogens increased prolactin secretion, while exogenous/endogenous androgens decrease prolactin secretion.

In further investigation, by examining the influence of testosterone and testolactone on modulation of prolactin in normal women, Serafini et al. [100] have concluded that inhibition of aromatization by testolactone resulted in a significant increase in prolactin, which was similar to the increase which occurred with testosterone when testolactone was not administered.

In vivo studies demonstrated that treatment with aromatase inhibitors significantly alters the endogenous endocrine profile, especially estradiol, without concomitant alteration in rat ovarian histology at concentrations slightly above physiologic levels [10].

In their work, Marynick et al. [101] have confirmed that testolactone itself did not influence gonadotropin levels. They maintained serum testosterone and estradiol at constant physiological levels with infusion of testosterone, and then compared serum luteinising hormone (LH) and follicle stimulating hormone (FSH) levels before and during testolactone and estradiol administration, and no differences were found. Gooren et al. [102] have investigated the impact of estrogens in the feedback regulation of gonadotropin secretion. They have studied basal and luteinizing hormone-releasing hormone (LRH)-stimulated gonadotropin in a group of orchidectomized male-to-female transsexuals on estrogen treatment. Agonadal and eugonadal subjects have received ethinylestradiol or testolactone or tamoxifene and the levels of LH and FSH secretion were measured. The data in this work do not provide direct evidence that estrogen negative feedback occurs at the level of the hypothalamus or pituitary, and the authors have concluded that this is not an LRH-mediated phenomenon, but rather the result of a direct effect of estrogens at the level of the gonadotroph.

Smals et al. [103] investigated effect of lowering estradiol levels by testolactone on basal and gonadotropin stimulated Leydig cell function in men. After testolactone therapy, the observed a lowering of circulating estradiol levels, the enhancing of the secretion of FSH, 17-hydroxyprogesterone, and a lowering of the degree of testosterone without affecting on serum LH levels. In addition, there was greater accumulation of 17-hydroxyprogesterone than of testosterone, suggesting 17,20-lyase inhibition. Although Vigersky et al. [19] demonstrated that testolactone has antiandrogenic properties, androgen receptor blockade cannot account for the unexpected accumulation of 17-hydroxyprogesterone over testosterone; the authors concluded that testolactone exerts its 17,20-lyase blocking effect through another mechanism. While the aromatase-inhibiting action of testolactone has been attributed to its competition with testosterone and also androstenedione for microsomal aromatase cytochrome P450 [2], Smals et al. [103] have speculated that testolactone, as a pseudo/substrate, also competes for the binding sites of cytochrome P450 17,20-lyase, and leads to inhibition of both key enzymes. Thus relief of the 17,20-lyase block induced by estradiol lowering might be obscured by a concomitant action of testolactone.

Zumoff et al. [104] have applied testolactone to confirm their hypothesis that obese men have elevated serum estrogen levels and diminished levels of FSH and free and total testosterone, all in proportion to their degree of obesity. The decreases in testos-

terone and FSH constitute a state of hypogonadotropic hypogonadism (HHG), and authors have concluded that it results from feedback suppression of the pituitary by the elevated estrogen levels.

Epilepsy is a disease that can be controlled with drugs nowadays. However, taking drugs to control epilepsy often leads to androgen deficiency in men, which can affect reproduction, lead to sexual dysfunction, and even possibly exacerbate seizure frequency. The assumption is that antiepileptic drugs affect the conversion of testosterone to estradiol. These side effects could be controlled by testosterone therapy, which could have a moderate beneficial effect on reproductive and sexual functionality. While testosterone is thought to reduce the frequency of seizures, estradiol, on the other hand, has the opposite effect. For this reason, Herzog et al. [105] in their research compared the effect of testosterone therapy with the effect when testosterone was given in combination with the aromatase inhibitor testolactone. They concluded that combined therapy leads to the normalization of estradiol levels, and to greater improvement in sexual function than testosterone alone, and also to a reduction in seizure frequency, due to reduced estradiol levels.

4.6. Fibromatosis

Sauven [106] published a case report of musculo-aponeurotic fibromatosis treated by surgery and testolactone. This was the first case in which testolactone therapy was administered after complete excision of the lesion. She observed no side effects with this treatment and there was no increase in tissue in ten months.

4.7. Atherosclerotic Cardiovascular Disease

It is known that homocysteine can promote atherosclerosis, while levels are associated with increased risk for atherosclerotic cardiovascular diseases. While total homocysteine (tHcy) levels are known to be higher in men than in women, Zmuda et al. [107] hypothesized that sex hormones may influence their levels. With the goal to examine the influence of androgens on increasing levels of tHcy, Zmuda et al., in addition to testosterone, applied testolactone in order to examine the effects of the conversion of androgens into estrogens in patients. Subjects received testosterone enanthate, testolactone, or both drugs together in a crossover design, but testolactone did not significantly influence tHcy levels.

4.8. COVID-19

In April 2020, World Health Organization (WHO) declared Corona Virus Disease (COVID-19) as a global pandemic disease, and since then the search for an adequate drug has been current in scientific circles. It is known that COVID-19 was caused by the SARS-CoV-2 virus, from a group of pathogenic coronaviruses. Mujwar [108] has investigated the triple mutant strain of SARS-CoV-2, which was more virulent and pathogenic than its original strain, with a high mortality rate in the second wave of the coronavirus disease. In order to develop a potent inhibitor, molecular docking simulation-based virtual screening of a ligand library consisting of FDA-approved drugs, followed by molecular dynamics simulation-based validation of leads, was performed. In this investigation, testolactone was among the top ten drug molecules with good binding energy, but further investigations were not pursued.

4.9. Side Effects

Selectivity of aromatase inhibition by therapeutic agents is crucial for their tolerability and the avoidance of unwanted side effects. The major side effects of testolactone are gastrointestinal [21,71,74,94]. Paresthesias, aches and edema of extremities, and nausea and vomiting, have occurred in a few patients during treatment with testolactone, while alopecia alone and alopecia with nail growth disturbance have also been reported [39].

In clinical tests, testolactone demonstrated a very low rate of toxic effects, consisting of occasional vomiting, frequent nausea, or increased frequency of diarrhea, with no side effects of an endocrine nature [11,59]. With increasing the dose of testolactone, the drug

did not cause new undesirable effects; further, there was a fall in urine calcium, and no hepatic, renal, or other toxicity, no change in serum high-density lipoprotein lipids, and no hypercalcemia [47].

In their dose response evaluation in advanced breast cancer, Volk et al. [109] concluded that testolactone is effective for the palliative primary treatment and has the unique advantage of having no significant metabolic, masculinizing, or feminizing hormonal activity. Testolactone was non-toxic and well-tolerated, where severe side effects were in the 2000 mg per day dose group and involved the gastrointestinal tract. The effect of testolactone on metabolic and serum lipid status was also investigated. Testolactone administration caused no protein anabolic or androgenic effects, while it lowered serum cholesterol and phospholipid levels, as well as α-lipoprotein lipids, and increased postheparin plasma hepatic triglyceride lipase activity (HTLA) [110–112].

5. Biologically Active Derivatives and Analogs of Testolactone

Testolactone has been a structural motif for the molecular design of numerous of novel molecules with potential biological activity. Although they were synthesized because of the good antitumor activity of testolactone, an additional reason was the excellent and diverse bioactivity of other steroidal lactones [113–118]. The aim of this section is to present steroidal compounds, classified as testolactone analogues, possessing the most interesting structures and good biological activity.

Hydrogenated precursor of testolactone **1** is a well-known compound testololactone (**2**, hydrotestolactone, D-homo-17a-oxaandrost-4-ene-3,17-dione, 3-oxo-13,17-secoandrost-4-ene-17,13α-lactone), which has also shown good antitumor potential that, additionally, confirmed the importance of D-homo lactone moiety as a pharmacophore. It was determined that testololactone (**2**) is a good inhibitor of aromatase, although less potent than testolactone (**1**) [52,53]. Because of that, testololactone has never been in clinical use. Nevertheless, research around the bioactivity of this testolactone analog did not stop, and it was determined that it also controls prolactin levels in males [119]. The newest findings regarding testololactone indicate its positive effect on the process of reverse transformation of cancer cells into healthy ones [120].

Furthermore, other D-homo lactone steroids have also exhibited significant antitumor activity. Some of them are shown in Figure 5. Compound **25** showed 72.5% of aromatase inhibition for 2 μM concentration [121], while compound **26** had good antiproliferative activity on HeLa cells (GI_{50} 28.2 μM) [122]. Structure containing D-homo lactone moiety and ester of *p*-bis(2-chloroethyl)aminophenylacetic acid on C3 (**27**), along with its lactam analogs **28** and **29,** are proven to be effective in causing markedly increased SCE (sister-chromatid exchange) rates and cell division delays in human lymphocytes [123]. In more recent research, compounds **27** and **28** showed good anticancer activity against pancreatic cancer cell lines [124,125]. The best GI_{50} of 8 μM is observed for D-homo lactam **28** on Hs776T cells.

Structures of some novel steroids with C3 ester groups and D-homo lactone ring **30–39** are presented in Figure 6 [126]. These compounds inhibit 50% of the activity of 5α-reductase in concentrations from 0.025 nM to 1300 nM. Steroid esters **30–39** do not bind to androgen receptors or have a very low relative binding affinity. In vivo tests showed that **34**, **37**, and **38** with high 5α-reductase inhibitory activity also decreased the weight of the prostate and seminal vesicles as compared to the testosterone-only treated animals, and that these steroidal lactones could have therapeutic potential for the treatment of androgen-dependent diseases.

Steroidal-methylene D- and A-lactones **40–43** (Figure 7) showed good inhibition of human nasopharyngeal carcinoma (KB) cell growth with LD_{50} of 0.72 μg/mL for **43** to 1 μg/mL for **40** [127]. This indicates that the lactone moiety in other rings of the steroidal core can be responsible for its bioactivity, which could also be the case with biologically potential of derivatives with a lactone in the B ring of the steroid core, also shown in Figure 7. Using a modified bean second internode bioassay, brassinolide activity was tested

on six new D-homo lactone derivatives **44–49**, and the B-homo lactone derivatives **48** and **49** have shown the highest activity [128].

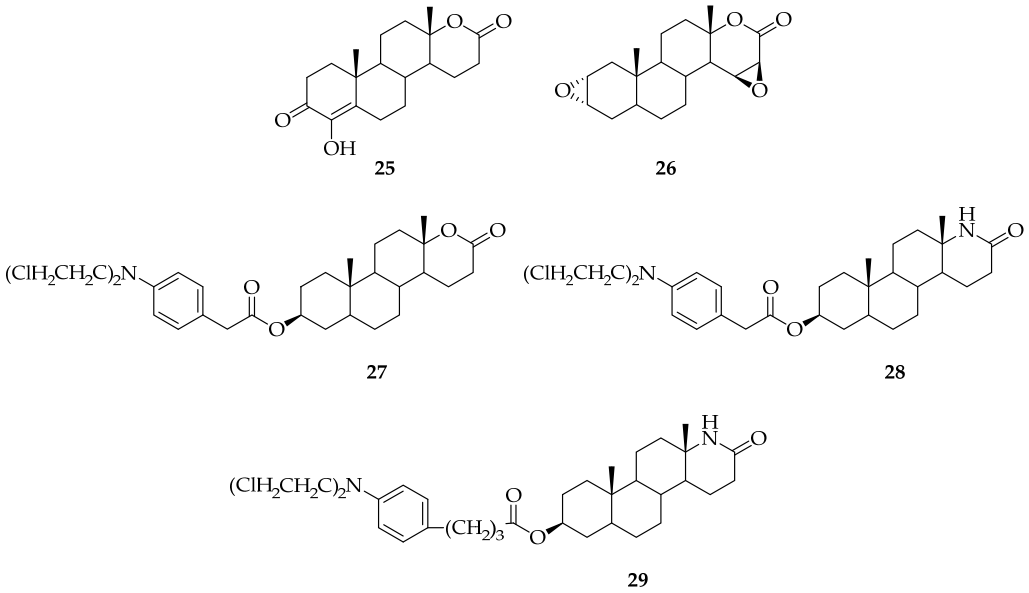
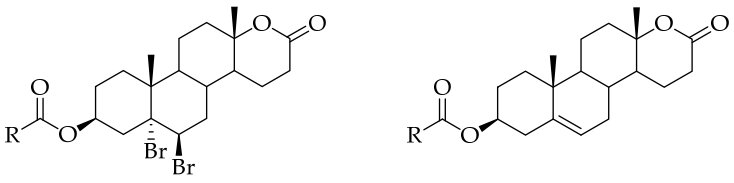


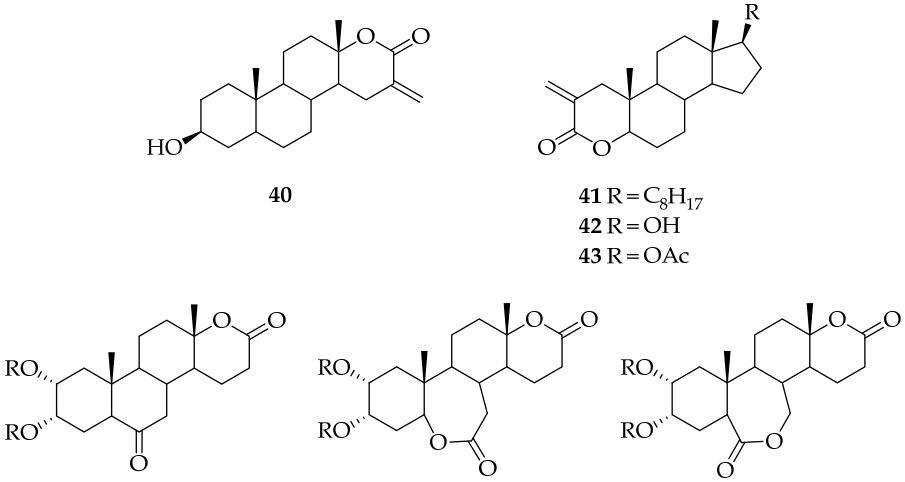
Figure 5. Structures of some D-homo lactone steroids with significant antitumor activity.



30 R = $CH_3CH_2OCH_2$
31 R = CH_3CH_2
32 R = $CH_3CH_2CH_2$
33 R = $CH_3CH_2CH_2CH_2$
34 R = $CH_3CH_2CH_2CH_2CH_2$

35 R = $CH_3CH_2OCH_2$
36 R = CH_3CH_2
37 R = $CH_3CH_2CH_2$
38 R = $CH_3CH_2CH_2CH_2$
39 R = $CH_3CH_2CH_2CH_2CH_2$

Figure 6. Structures of some biologically active steroids with C3 ester groups and D-homo lactone ring.



40

41 R = C_8H_{17}
42 R = OH
43 R = OAc

44 R = Ac
45 R = H

46 R = Ac
47 R = H

48 R = Ac
49 R = H

Figure 7. Biologically active steroidal-methylene and brassinosteroid with lactone ring.

The structure of testolactone and its biological activity were one of the motives in the design of a series of D-homo lactone steroids for our research group. These compounds have shown good bioavailability, antiproliferative activity, and inhibition of various enzymes involved in steroidogenesis [129–134]. Among the significant number of structures, compounds presented in Figure 8 stand out for their antiproliferative activity against different tumor cell lines [135–139]. High activity against estrogen-negative breast cancer cells (MDA-MB-231) was observed for compounds **50** (2.09 μM), **51** (9.30 μM), **54** (3.38 μM), **55** (4.40 μM), **56** (6.16 μM), **57** (4.18 μM), and **60** (9.13 μM), while on estrogen-positive breast cancer cells (MCF-7) only trihydroxy derivative **53** showed significant activity (8.62 μM). This compound was the most potent against lung fibroblasts (A549 0.99 μM), and, together with compounds **59** and **61,** was active on colon carcinoma cells (HT-29) with IC_{50} values of 9.34, 3.97, and 0.06 μM, respectively. Furthermore, significant activity was observed on the prostate cancer cells (PC3) for compounds **52** (2.64 μM) and **62** (2.18 μM), and cervix carcinoma cells for compounds **55** (7.06 μM) and **58** (4.97 μM). Compound **50** was also encapsulated in chitosan nanoparticles, and such particles have displayed strong cytotoxicity towards MDA-MB-231 and PC-3 cell lines with a lack of hormone activity, indicating their safety and efficacy [140].

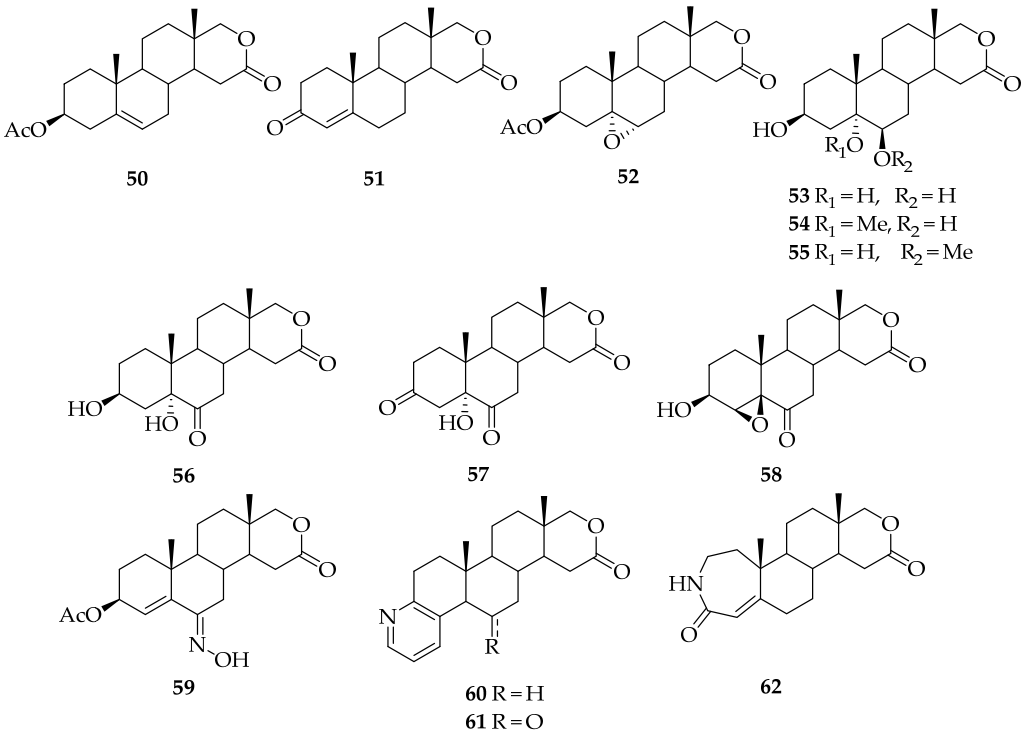


Figure 8. D-homo lactone steroids of with significant antiproliferative activity against different tumor cell lines.

6. Conclusions

Testolactone, marketed under the brand name TESLAC, is considered a pioneering drug for the treatment of breast cancer. It was first synthesized in 1953, and its use in the treatment of estrogen-dependent breast cancer started in 1970. The ability of testolactone to block estrogen production and prevent the growth of breast cancer cells by inhibiting the aromatase enzyme was discovered in 1975. It is a weak inhibitor, with a moderate clinical response, which led to its replacement by more potent aromatase inhibitors and withdrawn from use in 2008. Besides being able to lower estrogen levels in women and inhibit estrogen synthesis in human breast tumor tissue, also at a high dose, it has antiandrogenic effects, which were hypothesized to be that of a suicide action. Testolactone in therapy has been successful in minimizing virilization; the regressions produced were very satisfying and

free of toxic and hormonal effects. However, recent clinical trials do not exist, probably due to its low activity and the emergence of newer-generation aromatase inhibitors. The importance of testolactone is shown by the conducting of large-scale, high-quality studies on its use for the treatment of various diseases, such as breast cancer, prostatic carcinoma, human benign prostatic hyperplasia (BPH), desmoid tumors, carcinoma of the pancreas, precocious puberty, gynecomastia, etc. On the other hand, in the search for new drugs, particularly the development of new aromatase inhibitors, testolactone is often used as a starting compound for chemical modification to increase potency or lead compounds for the design of new more biologically active compounds.

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