A Trojan Horse in Drug Development: Targeting of Thapsigargins Towards Prostate Cancer Cells

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Abstract: Available chemotherapeutics take advantage of the fast proliferation of cancer cells. Consequently slow growth makes androgen refractory prostate cancer resistant towards available drugs. No treatment is available at the present, when the cancer has developed metastases outside the prostate (T4 stage). Cytotoxins killing cells irrespective of the phase of the cell cycle will be able to kill slowly proliferating prostate cancer cells. Lack of selectivity, however, prevents their use as systemic drugs. Prostate cancer cells secrete characteristic proteolytic enzymes, e.g. PSA and hK2, with unusual substrate specificity. Conjugation of cytotoxins with peptides, which are selective substrates for PSA or hK2, will afford prodrugs, from which the active drug only will be released in close vicinity of the cancer cells. Based on this strategy prodrugs targeted at prostate cancer cells have been constructed and evaluated as potential drugs for prostate cancer. The potency of the thapsigargins as apoptotic agents make these naturally occurring sesquiterpene lactones attractive lead compounds. Intensive studies on structure-activity relationships and chemistry of the thapsigargins have enabled construction of potent derivatives enabling conjugation with peptides. Studies on the mechanism of action of the thapsigargins have revealed that the cytoxicity is based on their ability to inhibit the intracellular sarco-/endoplasmtic calcium pump.

Key Words: Prostate cancer, targeted drugs, thapsigargin, PSA, hK2, Prodrug, SERCA.

1. RELATION TO PREVIOUS REVIEWS ON TARGETED DRUGS FOR TREATMENT OF PROSTATE CANCER

The present article reviews efforts to develop drugs that can be used for treatment of prostate cancer, in particular of metastatized prostate cancer (T4 phase), for which no treatment is currently available. Previous attempts to develop drugs targeted against prostate cancer cells based on analogues of thapsigargin have been reviewed by Denmeade and Isaacs [1, 2]. The present review, in particular, includes results obtained since 2005 and the chemistry behind the development of new and appropriate thapsigargin analogues. The chemistry of thapsigargin and other natural products from the genus *Thapsia* was last reviewed in 1997 [3].

2. PROSTATE CANCER

2.1. Epidemiology of Prostate Cancer

Among men prostate cancer is on a global basis the third most common cancer disease, and the leading cause of cancer disease in men in Europe, North America and some parts of Africa [4]. In Sweden a study revealed that in the period 1995 to 1999 the incidence of overt prostate cancer in men older than 85 years was 1.2%, and 0.7% for men older than 65 years. In Denmark the rate per 100,000 has increased from 30 to 40 per year in the period from 1995 to 2000, whereas during the same period the number has been constant or slightly declining in USA [5] and in British Columbia [6]. However, the prevalence is much higher when the early stages in prostate cancer development are included. Thus, an autopsy study of 600 men in Detroit, MI, USA, showed that 30% of all men in their 30s, 50% of men in their 50s and more than 75% of men in their 80s carried a latent prostate cancer [7]. The occurrence of prostate cancer varies considerably among ethnic populations and countries. Black men in the USA are the most vulnerable (1.4 - 2.0%), whereas the rate is only 0.002% for Chinese men in Tanjin [5, 7]. In the USA approximately 34000 men died from prostate cancer each year in the period 1990 to 1995 [5].

2.2. Clinical Diagnosis

Prostate cancer used to be diagnosed only when symptoms of advanced disease occurred or when patients were treated for presumed benign diseases such as a hypertrophied prostate gland. More cases are now detected because of screening programs in which the prostate specific antigen (PSA) level is measured. PSA is a proteolytic enzyme secreted into the semen by the epithelial cells of the prostate, with its physiological function being to regulate the viscosity of the ejaculate. Normally, small amounts of PSA leak into the circulation with reported concentrations of less than 4 ng/mL. A steady increase of PSA concentration beyond this level may indicate the presence of malignant prostate cells that upon successful treatment will be followed by a return to a normal concentration. Since PSA has been hypothesized to contribute to the development of prostate cancer, inhibitors of the protease are being developed for potential treatment of the disease [8]. The significance of the increased PSA level for diagnosis of prostate cancer is still being debated. Opponents argue that the purpose of screening should not be merely to detect a higher incidence, but to result in an active intervention such as "a radical prostatectomy" to reduce the mortality. A study encompassing 74,000 and 50,000 men, respectively, in order to compare the mortality among screened and unscreened populations is underway and the results are expected to be published in 2010 [9]. In addition to surgical intervention, attempts to develop noninvasive methods by applying genomic techniques for earlier and more specific diagnosis of prostate cancer are on going [10], but at present the only way to provide the most unequivocal information is to carry out biopsies. The extent (stage) of the disease is classified according to the localization and size of the tumors, T1 means the tumor is not visible by imaging but only realized by biopsy, T2 the tumor is confined within the prostate, T3 the tumor extends through the prostate capsule, and T4 tumors invade other tissues (the disease has become metastatic).

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2.3. Treatment of Prostate Cancer

Prostate cancer at the T1 or T2 stage is often successfully treated by radical prostatectomy or radiotherapy. In the Scandinavian countries, however, prostate cancer in these stages is not always treated, but the status of the patients is kept under close surveillance (watchful waiting). The rationale for non-intervention being that the side effects of treatment often exceed the benefits and that in particular older patients are likely to die from other diseases before symptoms from the prostate cancer become manifest [9]. Radical prostatectomy is the standard treatment for the T3 stage prostate cancer, in preference to external-beam radiotherapy which has a less convincing effect than in the case of T1 and T2 stage prostate cancers. However, radical prostatectomy is seldom used as a monotherapy for T3 stage cancer, but usually combined with radiotherapy. Neoadjuvant hormone therapy has sometimes been used prior to surgery or radiotherapy. It is, however, unclear if this treatment improves the life expectancy. The decision as to how to treat patients with T3 disease depends on age, general health, and 10-year life expectancy from death by other causes, as well as the urologist's evaluation of the benefits obtained from a radical treatment in reducing the symptoms of the tumor. Hormonal therapy, thus reducing the testosterone level is the mainstay treatment of prostate cancer in the T4 stage. The testosterone level may also be reduced by bilateral orchidectomy. Though this treatment offers immediately relief from metastatic pain many men find the prospect psychologically unacceptable. Analogues of luteinising-hormone have an equivalent effect to bilateral orchidectomy. This treatment, however, may result in an immediate rise in the testosterone level, which might be dangerous for some patients, and consequently should be neutralized by concomitant use of antiandrogens during the first weeks of treatment. In general prostate cancer tumors consist of a heterogeneous mixture of androgen-sensitive and androgen-refractory cells. Hormonal treatment induces apoptosis in androgen-dependent tumors, but will not affect the androgen-refractory cells. Consequently, hormone refractory tumors are bound to develop eventually. Attempts have been made to treat hormone refractory tumors with chemotherapeutics like doxorubicin (**1**), daunorubicin (**2**), fluorouracil (**3**), estramustine (**4**), paclitaxel (**5**), docetaxel (**6**), etoposide (**7**), mitoxantrone (**8**), vinblastine (**9**), or combinations of these, (Fig. **1**). It is fascinating to realize that a review published as late as 2007 states that natural products still are an important source for chemotherapeutics [11, 12] since the majority of **1** - **9** are either natural products or derived from natural products. Chemotherapeutic treatment may increase life expectancy in a subset of patients, however, when the disease reaches the hormone refractory stage it is uniformly fatal, since current treatments are unable to completely eliminate the androgen refractory prostate cancer cells.

The primary mechanism of action of doxorubicin (**1**), daunorubicin (**2**), and other antitumor anthracyclines is the formation of a ternary drug-DNA-topoisomerase II complex, in which the topoisomerase is covalently bound to the broken DNA strand. This complex will trigger apoptotic cell death [13]. Drugs belonging to the taxane group [paclitaxel = taxol (**5**) or docetaxel = taxotere (**6**)] promote polymerization of tubulin heterodimers to microtubules and thereby suppress dynamic changes in microtubules, leading to mitotic arrests [14]. Fluorouracil (**3**) acts in several ways but principally as a thymidylate inhibitor interrupting the enzyme, which is a critical factor for the synthesis of thymine and thereby for the generation of DNA. Etoposide (**7**), which was developed from the natural product, *epi*-podophyllotoxin, induces DNA breaks by interaction with DNA topoisomerase II [15]. Mitoxantrone is a type 2 topoisomerase inhibitor. Vinblastine (**9**) inhibits the assembly of tubulin into microtubules and, consequently, prevent the cell from undergoing division [16]. In conclusion, as appears from the above examples, most chemotherapeutics that have been used against prostate cancer act by interfering with the cellular pathways occurring during the mitotic phase.

2.4. Pitfalls in the Present Treatment of Hormone Refractory Prostate Cancer

The doubling time for prostate cancer cells *in vitro* has been estimated to vary from 30 days for metastatic prostate cells from hormonally untreated patients to 580 days for localized prostatic cancer cells within the prostate [17]. Cells isolated from bones of hormonally failing patients have doubling time of 94 days. All of these figures are so low as to indicate that no patient could be expected to live long enough to experience symptoms and die from the cancer [17]. Since patients actually do develop life threatening prostate cancer and a considerable number of patients die from the disease, the estimated doubling times clearly must be underestimates. On the other hand, the findings explain why only a fraction of men carrying latent prostate cancer actually have symptoms and most die from other causes before the cancer develops [7]. A further implication of the slow growth is that chemotherapeutics, which interfere with processes in the mitotic phase, will not significantly affect prostate cancer cells, since only a vanishing fraction of the cells are in this phase [17]. Accordingly, taxol and doxorubicin have been shown only to kill cells efficiently in high growth cell cultures, and much less efficiently in low growth cultures of the same cell type [18, 19]. In contrast a cell toxin like thapsigargin (**10**), which targets a pathway essential for survival, irrespectively of the phase of the cell cycle, is equally effective in high and low growth cell cultures [18]. A drawback of this approach, however, is that such cell toxins will kill benign as well as malignant cells.

3. PROPERTIES OF THE THAPSIGARGINS

3.1. Occurrence and Absolute Configuration of the Hexaoxygenated Thapsigargins

The thapsigargins are a group of naturally occurring sesquiterpene lactones only occurring in the genus *Thapsia* belonging to the family Apiaceae and in *Laser trilobum* (Apiaceae). According to Flora Europaea the genus *Thapsia* comprises three species *T. garganica* L.*, T. villosa* L., and *T. maxima* Miller [20]. However, later phytochemical and morphological studies and chromosome counting have questioned this simple division of the genus and suggested that additional species should be added and the botanical names revised [21-23].

Originally the thapsigargins were named after the plants, from which they first were isolated. After establishment of the structures, however, the new compounds all were named as thapsivillosins. Thus, thapsigargin (**10**, Fig. **2**) and thapsigargicin (**11**) were isolated from *T. garganica,* thapsitranstagin (**12**) from *T. transtagana,* the thapsivillosins A - E (**13** - **16**) and thapsivillosins G and H (**17** and **18**) from *T. villosa*, a plant species whose homogeneity has been questioned. Thapsivillosin I**,** J and L (**19**, **20** and **22**) all were isolated from *T. garganica,* whereas thapsivillosin K (**21**) was isolated from *T. transtagana*. Most of the compounds have later been found in additional species.

Characteristic for the hexaoxygenated thapsigargins is the presence of the 2β,3α,7β,8α,10β,11α-hexaoxygenated-6β,12-guaianolide nucleus. The pentaoxygenated thapsigargins are often mentioned as trilobolides (Section 2.2). The H1 atom is α in contrast to the stereochemistry at C1 of guaianolides isolated from the Asteraceae family [3]. The stereochemistry at C1 is of importance for the SERCA inhibitory activity. A characteristic feature for all sesquiterpene lactones from higher plants is the α-disposal of H7. In thapsigargin H7 has been substituted with a β-hydroxy group, which has changed the annelation between the cycloheptane and the lactone ring to a *trans*-annelation in contrast to the typical *cis*-annelation of the lactone ring in other 6,12-guaianolides from Apiaceae. The thapsigargins thus are distinguished by a densely oxygenated guaianolide nucleus and the stereochemistry at C7. The class is further characterized by the unusual fatty acids, with which the alcohol groups of the nucleus are esterified. The structures were originally based on NMR studies, which revealed the constitution of the
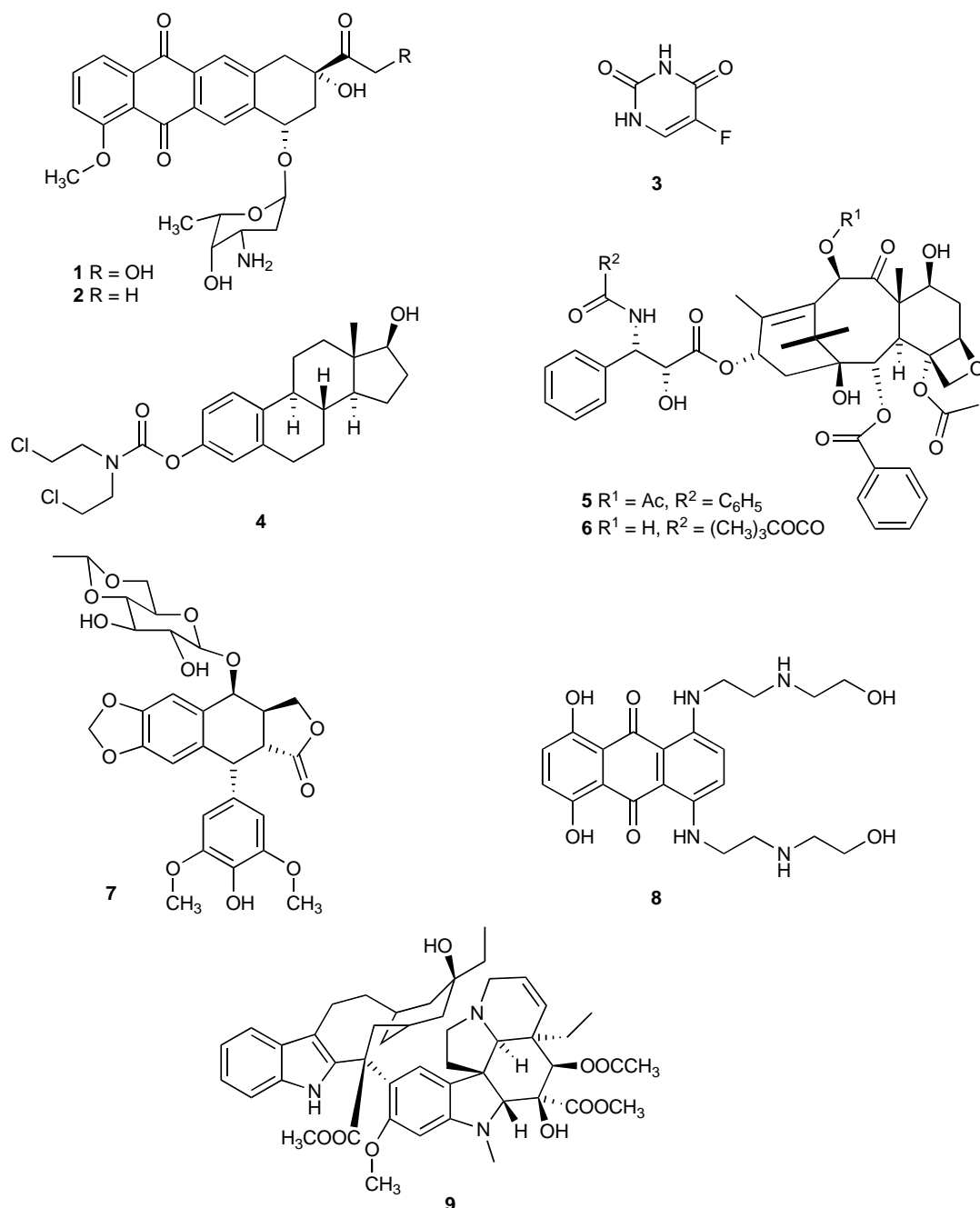
Fig. (1). Structures of the chemotherapeutics doxorubicin (**1**), daunorubicin (**2**), fluorouracil (**3**), estramustine (**4**), paclitaxel (**5**), docetaxel (**6**), etoposide (**7**), mitoxantrone (**8**), and vinblastine (**9**) used for treatment of androgen refractive prostate cancer.

molecules. The relative configuration was established by an X-ray study of the epoxide (**24**, Fig. **3**) formed by treatment of thapsigargin with thionyl chloride. Only very few similar transformation of a vicinal diols into an epoxide by treatment with thionyl chloride are reported. The different acyl residues were located by MS studies [24]. Finally, the absolute configuration was established by taking advantage of the coupled exciton coupling between the conjugated double bonds in angelic acid and the C4-C5 double bond. The suggested structures have been confirmed by total syntheses and by X-ray studies of thapsigargin bound to SERCA [25, 26]. In the case of thapsivillosin C (**14**) synthesis also has established the absolute configuration of the 2-methylbutanoyl residue as *S* [27].

All the thapsigargins and the trilobolides are very potent SERCA inhibitors, but most pharmacological studies have con-

cerned thapsigargin, which is present in up to 1% of the dry weight of roots or fruits of some types of *Thapsia garganica*.

3.2. Occurrence and Absolute Configuration of the Pentaoxy-genated Thapsigargins: The Trilobolides

The trilobolides are characterized by the 3α,7β,8α,10β,11α-pentaoxygenated 6β,12-guaianolide nucleus with a *trans*-annelated lactone ring. The trilobolides thus possess the same substitution at the guaianolide nucleus as the hexaoxygenated thapsigargins except for the absence of the 2-oxy substituent. The first characterized representative of the trilobolides (Fig. **4**), trilobolide (**26**) was isolated from *Laser trilobum,* (Apiaceae), and consequently the trilobolides in contrast to the thapsigargins have also been found in species not belonging to the genus *Thapsia*. At the present however,
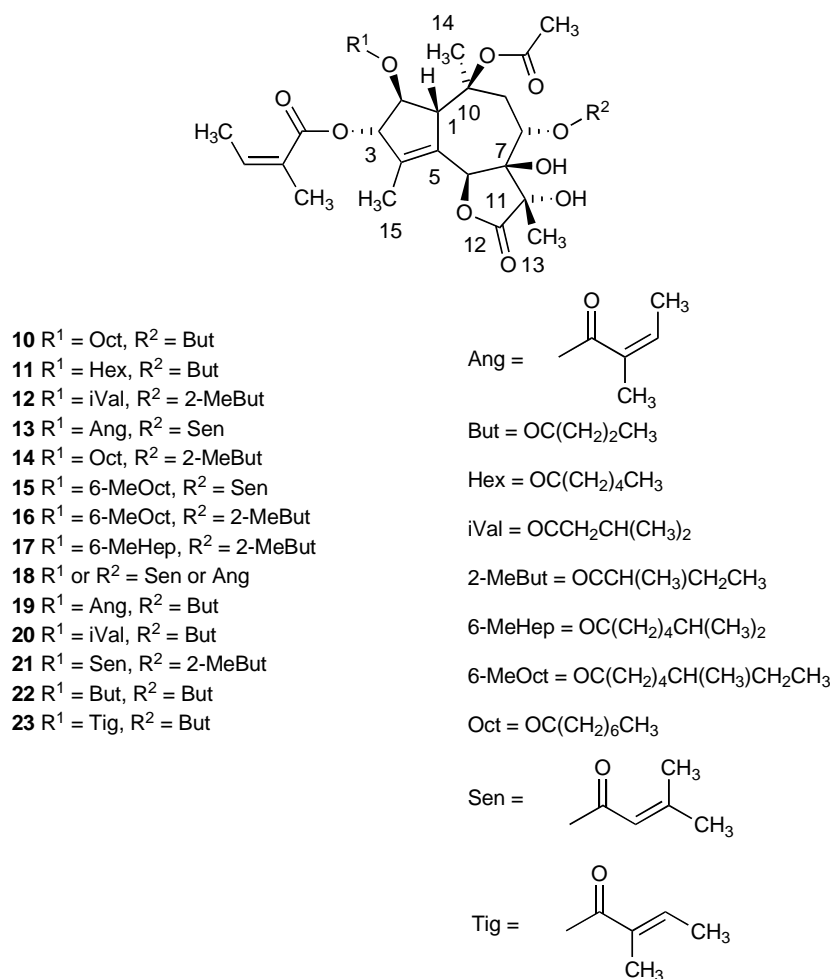
Fig. (2). The absolute configurations of the hexaoxygenated thapsigargins thapsigargin (**10**), thapsigargicin (**11**), thapsitranstagin (**12**), thapsivillosins A - M (**13** - **23**).
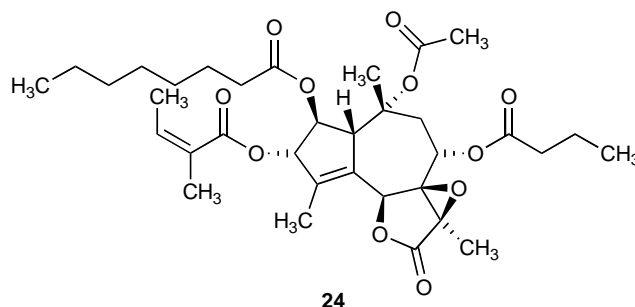

24

Fig. (3). The absolute configuration of the epoxide **24** formed by reacting thapsigargin with thionyl chloride.

Laser trilobum is the only species outside the genus *Thapsia,* in which thapsigargins including trilobolides have been detected. Nortrilobolide (**25**) was isolated from *T. garganica,* and thapsivillosin F (**27**) from *T. villosa.* Also in this case, the compounds have been found in other species belonging to *Thapsia.*

3.3. Chemistry of the Thapsigargins

3.3.1. Deacylation

Most of the chemistry of the thapsigargins has been made using thapsigargin as a model. The *cis* orientation of the C11 hydroxy group and the butanoylate at O8 makes the compound very sensi-

tive to solvolysis probably because of anchimeric assistance from OH11. The O8-debutanoyl derivative **28** is formed if a methanolic solution of the compound is left for a week at room temperature. This reaction is catalyzed by base and thapsigargin is converted into the debutanoylderivative in a few hours if triethylamine is added to a methanolic solution (Fig. **5**) [28]. Strengthened reaction conditions lead to further deacylations to give **29**. Thapsigargins with more sterically hindered acyl groups at O8 like 2-methylbutanoyl are less sensitive towards solvolysis.

The selective deacylation at O8 has been taken advantage of for the preparation of prodrugs [29]. Treatment of thapsigargin with a
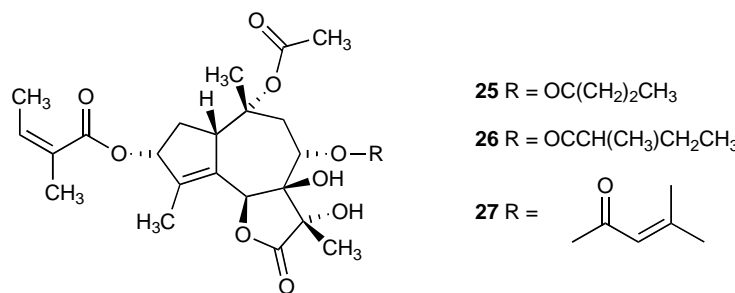
25 R = OC(CH$_2$)$_2$CH$_3$

26 R = OCCH(CH$_3$)CH$_2$CH$_3$

27 R =

Fig. (4). Absolute configuration of trilobolide (**26**), nortrilobolide (**25**) and thapsivillosin F (**27**).
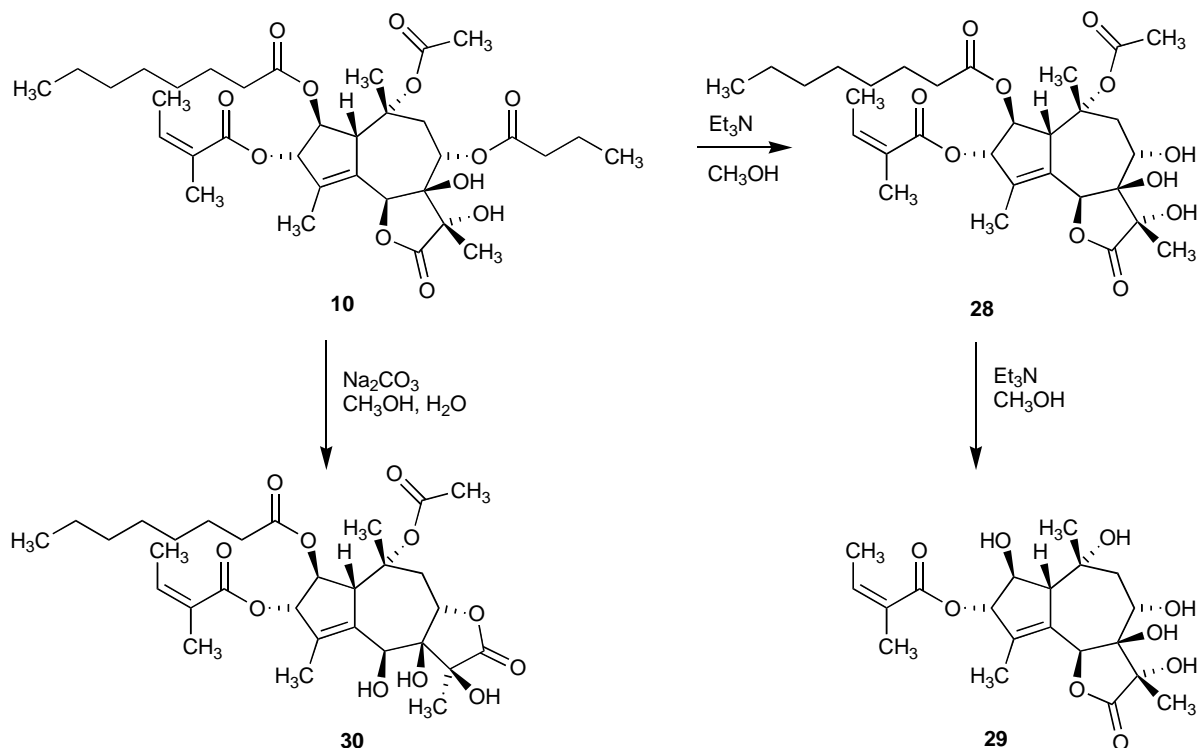

Fig. (5). Debutanoylation and relactonization of thapsigargin.

stronger base like sodium carbonate causes the lactone to open and after addition of acid to relactonize, gives a mixture of **28** and the 8,12-guaianolide **30**. The relactonization causes a major change of the conformation of the cycloheptane ring in **30,** evidenced from the change of the coupling constants between H8 and the two non-equivalent protons at C9 [30].

Reaction with thionyl chloride converts thapsigargin into the epoxide **24** as mentioned in section 2.1. Selective deacylation of the angeloate group has been obtained by oxidation with potassium permanganate to give the pyruvate ester **31**, which is selectively cleaved by treatment with pyridine in methanol (Fig. **6**). The subsequent alcohol **32**, however, is unstable and extensive transesterification is observed [31].

Some other selective transformations of thapsigargin have recently been reviewed [3].

3.3.2. Reduction of the Lactone

Selective reduction of the carbonyl group of the lactone i achieved by reduction with sodium borohydride or sodium bis(methoxyethoxy)ethoxyaluminium hydride to obtain a mixture of the α− and β-lactol **33** and **34**, respectively (Fig. **7**) [32]. Treat-

ment of the lactols with diethylorthoformate affords the two possible cyclic orthoformates **35** and **36** [32]. Treatment of the lactols with ethanethiol in hydrochloric acid yields the thioether, which may be reduced to **37** [33]. Since the latter reduction proceeds through a radical path the reaction also involves an isomerization of the angeloyl residue to a tigloyl residue.

3.4. Total Synthesis of the Thapsigargins

Even though the biological community reacted rapidly the properties of thapsigargin and an impressive numbers of biological studies were initiated with analogues of thapsigargin, the synthetic community reacted very slowly, so it was not until 2003 that the first paper on a successful synthesis of the trilobolides appeared [34]. In the synthetic field the dense population of functional groups caused unexpected reactions, e.g. an unprecedented catalytic selenium elimination, which afforded the C4-C5 double bond [34]. Although the mechanism behind this unexpected rearrangement has been interpreted [35] it delayed the successful total synthesis of some thapsigargins until 2007 [27, 36]. The 42 steps involved in the synthesis still makes isolation of thapsigargin from the fruits of *T. garganica* currently the only feasible way to obtain the compound
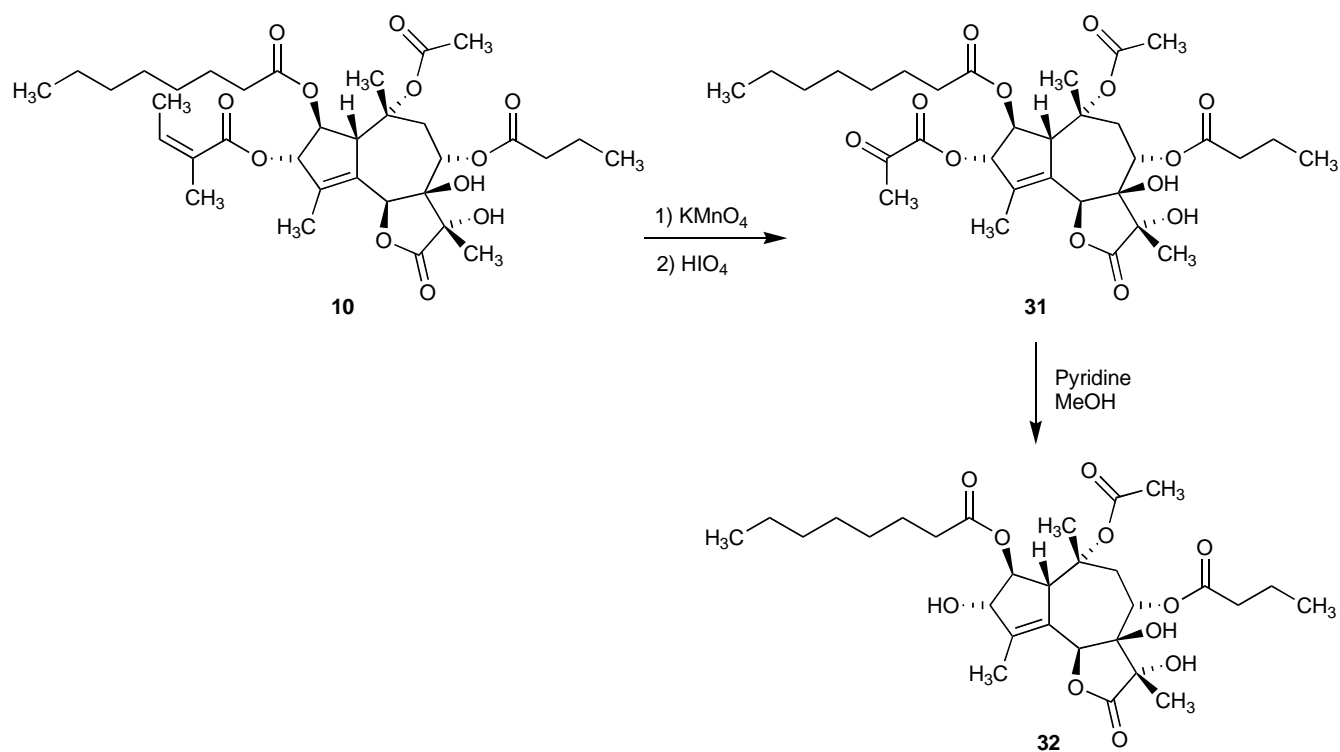
Fig. (6). Selective removal of the angeloyl residue from thapsigargin (**10**).
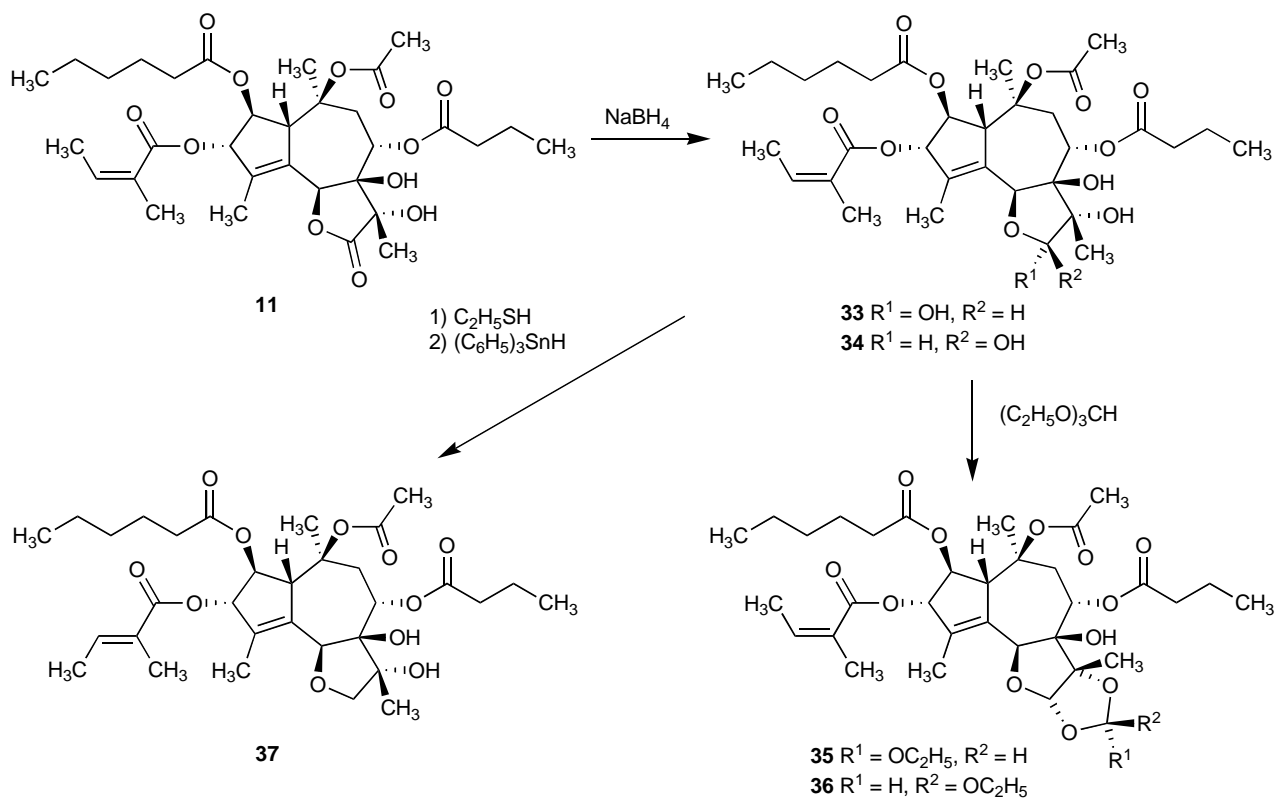

Fig. (7). Reduction of the lactone carbonyl group.

in large amounts, but beside the impressive academic achievement that it represents, the synthesis is interesting because it affords access to thapsigargin analogues with major changes in the skeleton

enabling elaborate structure-activity studies. As an example the intermediate **38** was converted into the analogs **39** and **40** (Fig. **8**) [37].
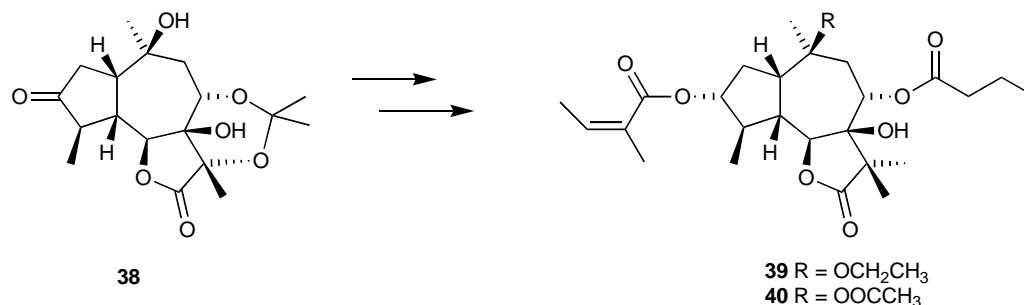
Fig. (8). Absolute configuration of the analogues (**39**, **40**) prepared by total synthesis.

3.5. Pharmacological Activities of the Thapsigargins

The resins of the roots of *T. garganica* have for centuries been used as "counterirritants" for relief of rheumatic pains because of their skin irritant properties. Theophrastos (372-287 BC) described the use of this drug which continued until the end of the 20th century. The drug was included in some pharmacopoeias including the French Pharmacopoeia from 1937. The two major active principles, thapsigargin and thapsigargicin were not isolated and structure elucidated until the 1970s [38].

3.5.1. In Vivo Studies of the Thapsigargins

The lethal dose of thapsigargin by intraperitoneal injection into mice was determined to be 1.8 mg/kg, and 0.8 mg/kg after invtravenous injection [39]. Intracutaneous injection into humans revealed that thapsigargin was equipotent to histamine in eliciting erythema. Treatment with an antihistamine diminished the flare [38]. Thapsigargin as well as thapsigargicin and thapsitranstagin also were shown to induce irritation of the mouse ear. In contrast thapsigargin-epoxide (**24**), the derivatives 8-O-debutanoylthapsigargin (**28**), and *iso*-thapsigargin (**41**, Fig. **9**) showed no skin irritating effect [40]. This finding inspired testing to determine if thapsigargin is a tumor promoter. Continued administration of thapsigargin to the skin of shaved mice pretreated with 7,12-dimethylbenz(a)-anthracene caused appearance of skin tumors and thapsigargin was classified as a non-12-O-tetradecanoylphorbol-13-acetate (TPA) tumor promoter. However, tumors only appeared if the mice were pretreated with TPA [40].

3.5.2. Effects of Thapsigargin on Tissues

In the concentration range 0.01 to 10 μM thapsigargin has a dual effect on rat thoracic aorta [41, 42]. Thapsigargin induced a concentration dependent increase in basal tone in normal physiological salt solution; whereas thapsigargin in potassium preconcentrated aortic rings caused a concentration dependent relaxation. Removal of the epithelium totally abolished the relaxant effect of thapsigargin and increased the contractile response. Probably the relaxing effect of the epithelium is mediated through endothelium-

dependent relaxing factors. Similarly, thapsigargin like the calcium agonist Bay K 8644, increased the spontaneous mechanical activity in rat portal veins without changing the frequency. Incubation with the calcium antagonist nitrendipine neutralized the effect of thapsigargin. The results indicate that thapsigargin exerts its effect by mobilizing extracellular as well as intracellular Ca^{2+} pools.

3.5.3. Effects of Thapsigargin on Cells

The finding that the flare produced by thapsigargin was diminished by administration of antihistamines inspired investigation to see if thapsigargin released histamine from mast cells. Thapsigargin was found to potently induce a histamine release if Ca^{2+} was present in the incubation medium [38, 43]. Later studies revealed that release of mediators was not restricted to mast cells, but a series of cells involved in the immune response could be induced to release mediators [44]. This study also gave the first clue to an understanding of the mechanism of action, since the mediator release was found to be preceded by an increase of the cytosolic Ca^{2+} level. Thapsigargin also affected the arachidonic acid metabolism [45, 46]. Furthermore, thapsigargin and TPA were synergistic [47].

Later studies revealed that prolonged incubation of cells with thapsigargin induced apoptosis of the cells in a proliferation independent manner [48, 49]. In contrast to chemotherapeutics like taxol and doxorubicin, which target processes in the proliferating cells and consequently only induce apoptosis to a minor extent in slowly proliferating LNCaP cells, thapsigargin kills these cells in the same concentration range, irrespective of rate of proliferation [18]. The induced apoptosis is dependent on extracellular Ca^{2+} since incubation with calcium chelators prevented apoptosis [50].

The Ca^{2+} gradient (low concentration in the cytosol and a high Ca^{2+} concentration in the endo/sarcoplasmic reticulum) is maintained by the SERCA pump which continuously transports Ca^{2+} from the cytosol into the reticulum. The cornerstones constituting the functional cycle have been characterized by X-ray analysis of the pump co-crystallized with ligands that lock the protein into these four conformations:
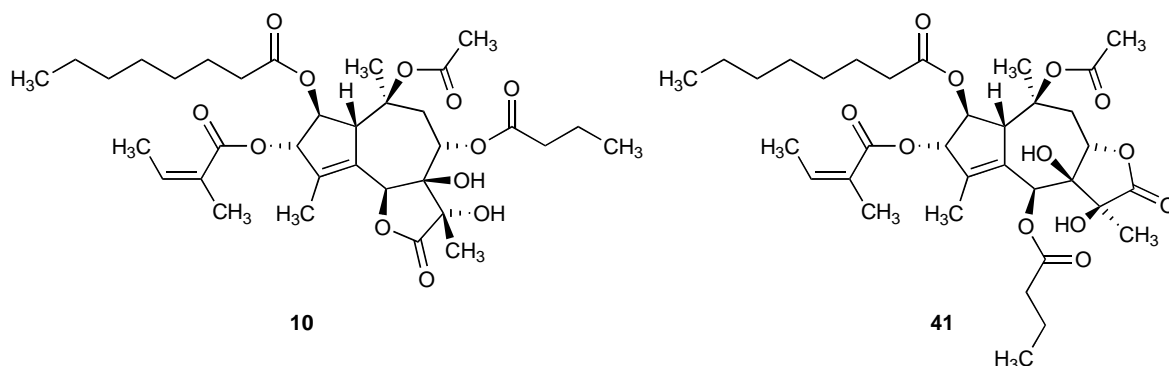

Fig. (9). Absolute configuration of thapsigargin (**10**) and *iso*-thapsigargin (**41**).

1) the E2-ATP form with the intramembranous cation binding sites oriented towards the cytosol to which, in the absence of Ca^{2+}, 2-3 protons are bound [51],

2) the $Ca_2E1\sim P$-ADP conformation, which is formed by phosphorylation with ATP after binding of two Ca^{2+} to the E2-ATP form. Ca^{2+} in this stage is present in an occluded form inside the membrane [52]

3) the E2P conformation, where the pump translocates the two occluded Ca^{2+} ions and releases them towards the lumenal side in exchange for 2-3 protons [53], and

4) the E2-P*-ATP transition state, where the pump has occluded the protons and is dephosphorylated to complete the cycle and reform E2P-ATP [53].

The lipophilicity of thapsigargin enables the molecule to penetrate the cell membrane and diffuse into the SERCA calcium pump, locking it into the E2 conformation. Locking of the SERCA pumps prevents it from maintaining the calcium homeostasis within the cell, which is essential for the survival and function of the cell (Fig. **10**) [54, 55].

3.5.4. Cell Death Induced by Thapsigargin

Thapsigargin provokes the apoptotic cell death pathway, which is which is characterized by membrane blebbing, cytoplasmic shrinkage, chromatin condensation, exposure of phosphatidylserine on the surface, and finally formation of apoptotic bodies [56]. From a therapeutic point of view formation of apoptotic bodies are advantageous since these are engulfed by neighboring cells by phagocy-
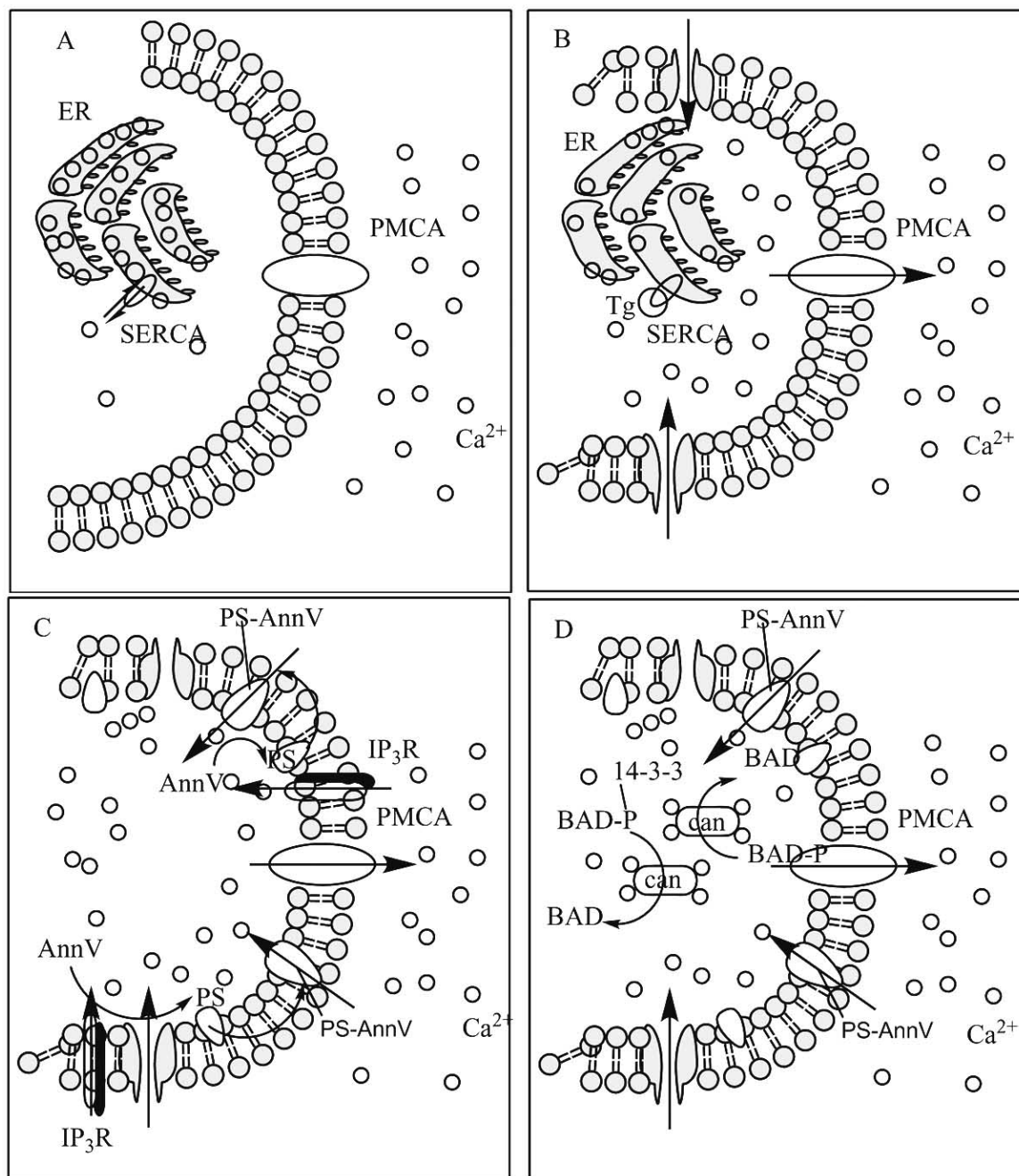

Fig. (10-1). The apoptotic process initiated by inhibition of SERCA. For a discussion see paragraph 3.5.4.
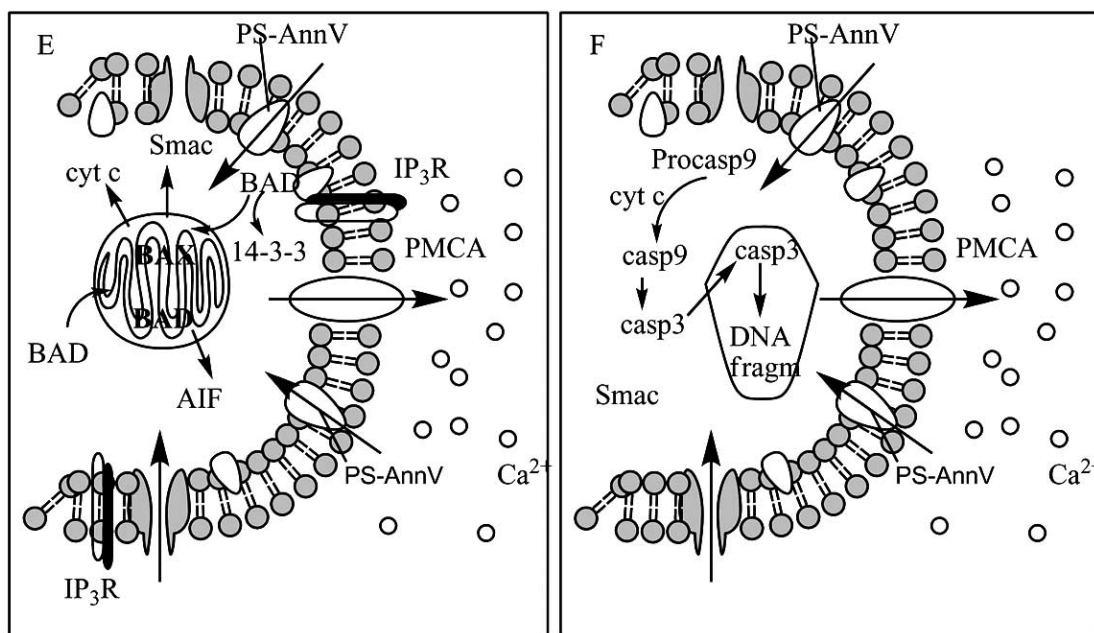
Fig. (10-2).

tosis without activation of the immune system. Alternative cell death pathways are autophagy, mitotic catastrophe, and necrosis [56]. Thapsigargin initiates apoptosis by inhibiting SERCA.

When the SERCA pump is deprived of the ability to remove Ca^{2+} (o) from the cytosol, passive leakage from the endoplasmic reticulum rises the cytosolic Ca^{2+} concentration to a level, which generates a signal changing the plasma membrane permeability, thus allowing an influx of Ca^{2+} from the extracellular medium, where the normal Ca^{2+} concentration is 1 - 3 mM [57, 58]. This Ca^{2+} entrance process operating through the Orai1/STIM factors, localized to the plasma membrane and ER, respectively, results in transfer of extracellular Ca^{2+} to the ER [59, 60] and an increase in the cytosolic Ca^{2+} level , e.g. from 20 - 70 nM to 200 - 400 nM within minutes after incubation of prostate cancer cells with thapsigargin in a concentration of 100 nM [48]. This causes an increased transcriptional and translational expression of a series of genes/gene products e.g. GAdd 153, calmodulin. After approximately 12 hours of increased cytosolic Ca^{2+} level activation of the calmodulin-dependent plasma membrane Ca^{2+} pump (PMCA), which is not affected by thapsigargin, decreases the cytosolic Ca^{2+} level to baseline level of 20 - 40 nM. During the return to baseline level an enhanced synthesis of proteins including the IP_3 type-3 receptor (IP_3R) occurs. The newly synthesized IP_3 receptor migrates to the plasma membrane and forms a Ca^{2+}-channel, through which extracellular Ca^{2+} flow into the cell (Fig. **10C**). During this second rise of the Ca^{2+} level, which occurs more than 24 h after thapsigargin exposure, the concentration rises to 200 - 400 nM. The second rise of the cytosolic Ca^{2+} level induces translocation of Annexin-V (AnnV) protein from the cytoplasm to the intracellular side of the plasma membrane and binds to phosphatidyl serine (PS). One of the characteristics of the apoptotic cell death pathway is the presence of the AnnV-PS complexes [56]. The AnnV-PS complex flips to the extracellular side of the plasma membrane and forms Ca^{2+}-channels, which allow an enhanced influx of Ca^{2+} causing a cytosolic concentration of 10 - 20 µM (Fig. **10C**) [1]. The enhanced cytosolic Ca^{2+} concentration is followed by the morphological changes that precede apoptosis. No apoptosis is observed if the extracellular Ca^{2+} concentration is kept at a level (e.g. 100 nM) preventing a µM cytosolic concentration. The cytosolic µMCa^{2+} concentration leads

to saturation of calmodulin and calcineurin (can) with Ca^{2+}. The saturated calcineurin complex dephosphorylates the pro-apoptotic protein BAD. Dephosphorylated BAD dissociates from the 14-3-3 protein, to which it is bound under physiological conditions, and translocates it to the mitochondria (Fig. **10D**) [1, 56]. In the mitochondria BAD displaces the proapoptotic protein BAX, which normally is bound to the apoptotic inhibitory proteins bcl-2 or bcl-x. BAX then interferes with the voltage-activated channel to form a leak in the membrane, through which cytochrome c (cyt c), Smac, and Apoptosis Initiating Factor (AIF) can be released to the cytosol (Fig. **10E**). On binding of cytosolic cytochrome c to the apoptotic activation factor complex, the complex enzymatically converts the procaspase 9 (procasp9) subunit within the complex to active caspase 9 (casp9). The cytosolic Smac prevents the Inhibitors of Apoptosis Proteins from inhibiting the activated caspase 9. Caspase 9 initiates a cascade reaction activating a number of other caspases, including caspase 3 (casp3). After penetration into the nucleus caspase 3 hydrolyzes the 45 kDa inhibitory subunit of the DNA fragmentation factor complex. This allows the 40 kDa DNAse subunit to be liberated in an enzymatically active form, which contributes to the degradation of the genomic DNA (Fig. **10F**). This degradation is further facilitated by Apoptosis Initating Factor released from the mitochondria. The result is the degradation of the nucleus and cell resulting in fragmentation into apoptotic bodies [61]. For a detailed discussion of the apoptotic process see Refs. [1, 56, 62-65].

3.6. Molecular Target of Thapsigargin

All biological effects of thapsigargin are preceded by an increased cytosolic Ca^{2+} concentration in the affected cells. This finding was rationalized when it was discovered that thapsigargin is a specific inhibitor of the sarco-endoplasmic reticulum calcium transport ATPase (SERCA) [54, 66, 67]. The SERCA family includes the products of three genes named SERCA1 (*ATP2A1*), SERCA2 (*ATP2A2*), and SERCA3 (*ATP2A3*), each giving rise to alternatively spliced mRNA [68]. Currently the SERCA family comprises 14 isoforms: two SERCA1 (1a and 1b), three SERCA2 (2a, 2b, and 2c), and in humans six SERCA3 (3a, 3b, 3c, 3d, 3e, and 3f). In some species (mice and rats) other specific isoforms of SERCA3

have been found [68]. Thapsigargin is a potent inhibitor of all three families of SERCA even though the inhibition constants, K_D, vary from 0.2, 1.3 and 12 nM towards SERCA1b, SERCA2b, and SERCA3a, respectively [69]. In all three cases the enzyme can be fully inhibited by low concentrations of thapsigargin. The binding site of thapsigargin has been characterized through X-ray analyses of the SERCA1a bound to thapsigargin and found to be located in a cavity formed between the M3, M5, and M7 transmembrane segments [25, 70]. A GRID analysis of the binding site surprisingly revealed that no hydrophilic interactions between the site and the highly oxygenated thapsigargin seem to be of importance. In contrast five regions enable favorable lipophilic interactions with thapsigargin (Fig. **11**). The GRID model thus suggests a pharmacophore model for the binding site of thapsigargin, comprising five lipophilic groups with three major sites located at the butanoyl, the acetyl, and the angeloyl, and two minor ones at Me15 and the octanoyl residues. Acetylation of the two hydroxyl groups only decreases the potency by a minor extent (**48**, **49**, and **50,** Table **1**) confirming that hydrophilic interactions only have a minor importance. The low importance of hydrophilic interactions was supported by a docking study, in which some of the analogues shown in Table **1** were docked into the binding site of thapsigargin. The model also could explain the observed trends of the potencies [71]. The importance of the lipophilic binding site L5 is illustrated by the pronounced difference in activity between thapsigargin and compound **44**. The stereochemistry at C3 and C8 is crucial for the activity as illustrated by the diminished activities of compound **46** and **47**. Two explanations might be offered for this finding, either the butanoyl group in **47** or the octanoyl group in **46** is displaced in a position which prevents them from interacting with L3 and L1 or steric interactions between the acyl groups and SERCA prevent binding. Previous empirical studies have revealed that a long flexible acyl group at O8 did not decrease the ability of ligands to bind to this binding site even though this acyl group terminated in a bulky substituent [29]. An X-ray analysis of the 8-*O*-(12-tert-butoxycarbamidododecanoyl)-8-*O*-butanoylthapsigargin (the Boc derivative of **53**) revealed that the guaianolide was bound at the thapsigargin binding site, whereas the long side chain was twisted behind the third transmembrane segment and the terminal tert-butoxycarboxamido groups appeared above one of the Ca^{2+} binding sites near the cytoplasmic surface (Fig. **11**) [37].

The structure also rationalizes the empirically obtained structure activity relationships obtained previously (Table **1**).

4. TARGETING THE THAPSIGARGINS

The selectivity of the majority of chemotherapeutics is based on the rapid proliferation of cancer cells making them sensitive to blockage of pathways involved in the mitotic phase. In contrast thapsigargin blocks the SERCA pump, the function of which is essential for quiescent as well as the proliferating cells. The drawback of this target, however, is that the function of the SERCA pump is crucial for benign as well as malignant cells. As a consequence thapsigargin exhibits a considerably toxicity after systemic administration [39]. Consequently drugs affecting the SERCA pump need to be targeted at the prostate cancer cells in order to avoid unacceptable side effects (Fig. **12**). The mechanism of action of thapsigargin clearly indicates that the molecule has to penetrate the plasma membrane to reach SERCA located on the endo- or sarcoplasmic reticulum membranes. The lipophilicity of the membrane indicates that conjugation of thapsigargin with a hydrophilic residue, e.g. a peptide, would prevent penetration of the plasma membrane and thereby neutralize the toxicity. The toxicity of the complex would be regained after cleavage of the hydrophilic residue to give an active agent.

4.1. Prostate Tissue Characteristic Proteases

A number of cells in the body secrete the proteolytic enzymes prostate specific antigen (PSA, sometimes referred to as human

glandular kallikrein 3, hK3) and human glandular kallikrein 2 (hK2). However, for both enzymes the amounts secreted in the prostate are orders of magnitude larger than those in other tissues [72]. The ability to secrete PSA and hK2 is preserved in benign prostatic hyperplasia as well as malignant prostate metastases [73, 74]. The intensity of hK2 expression in malignant cells was even found to increase, whereas the intensity of PSA was found to be highest in benign cells [75-77]. The physiological importance of the enzymes especially for non prostate tissue is not obvious, but upon mixing with seminal fluid PSA is activated and degrades the gel forming proteins semenogelin I and II. Degradation of semenogelin I and II affords liquefaction of the semen and thereby increases the motility of spermatozoa [72]. After secretion from prostate tissue PSA eventually enters the blood where it is inactivated by binding to and degradation by the major serum protease inhibitors α_1-antichymotrypsin, α_2-macroglobulin, and other proteins [73, 78, 79]. Like PSA, hK2 is also inactivated by binding to proteins in serum, the most important ones being α_1-antichymotrypsin, α_2-antiplasmin, antithrombin II, protein C inhibitor, and α_2-macroglobin [80, 81].
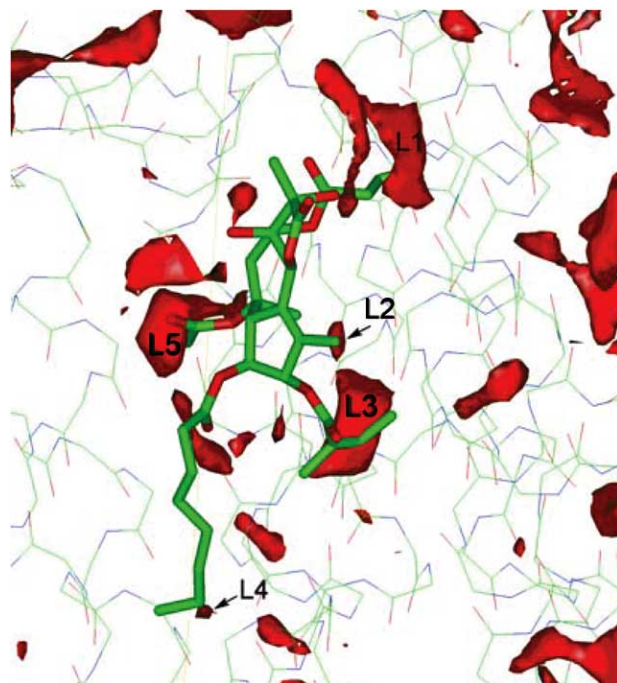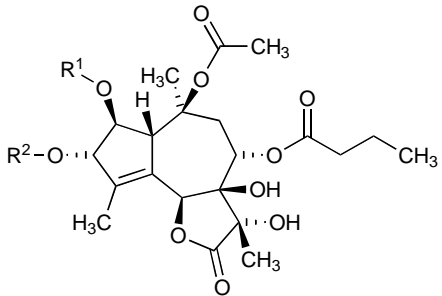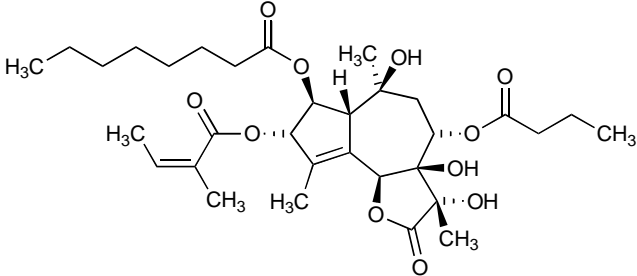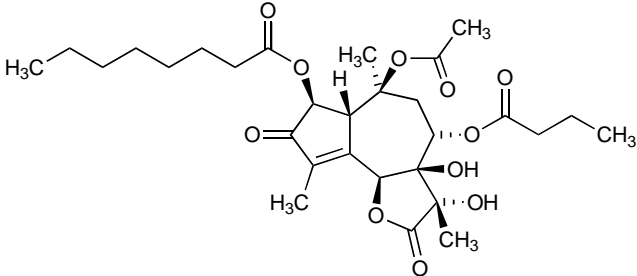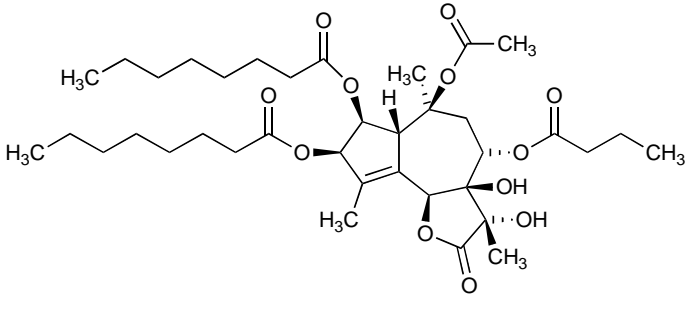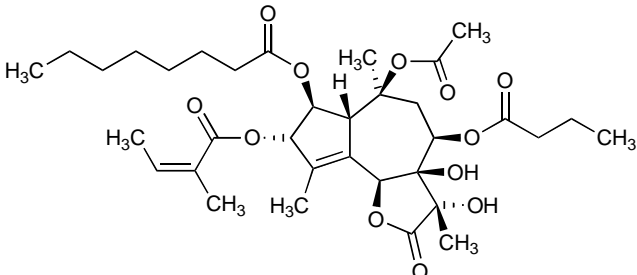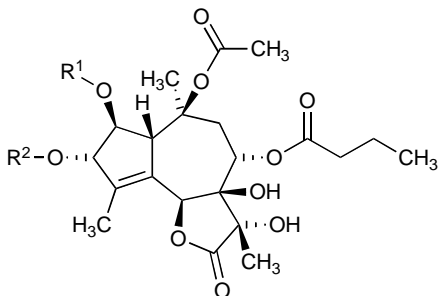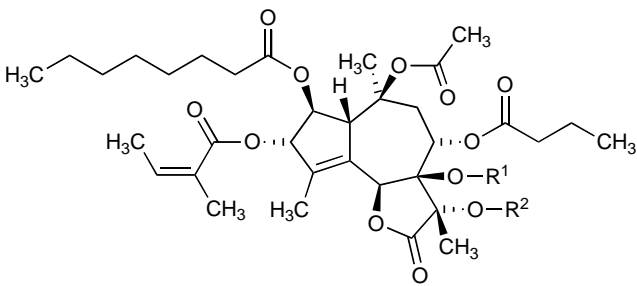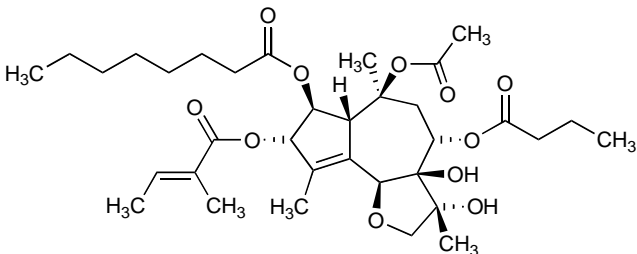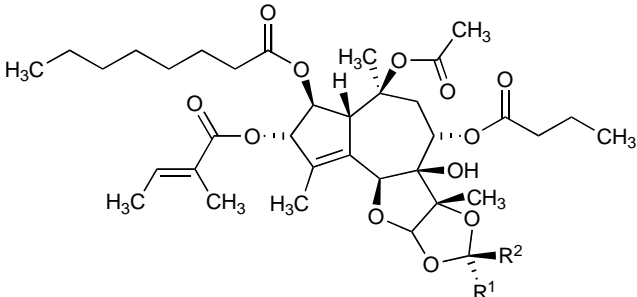

Fig. (11). Molecular interaction fields between thapsigargin and SERCA calculated for a methyl probe and contoured at -3 kcal/mol. Important lipophilic interactions occur between the butanoyl residue and region L1, a minor interaction between Me15 and L2, interaction between the angeloyl residue and L3, a minor interaction between octanoyl and L4, and an interaction between acetyl and L5 (reproduced with permission from J Med Chem [37]).

The substrate specificity of hK2 was investigated by analyzing the cleavage products of semenogelin I and II [80]. The enzyme preferentially cleaves peptide bonds C-terminal to arginines, but C-terminal cleavage after His(101) is also observed in semenogelin II. Consequently hK2 possesses trypsin-like substrate specificity. Studies with other substrates revealed that amino acids at some distance from the cleavage site also were of importance [80]. In contrast to hK2, PSA possesses chymotrypsin-like substrate specificity, even though the two enzymes are 78% homologous [81]. PSA cleaves on the C-side of most Tyr residues in semenogelin I and certain Leu residues, but not on the C-site of Phe and Trp site [82, 83].

Table 1. **Potencies of Analogues Relative to Thapsigargin (A Large Ratio Implies Poor Activity)**

Cpd			IC$_{50}$(A)/IC$_{50}$(Tg)
10	R^1 = Oct	R^2 = Ang	1
42	R^1 = Oct	R^2 = Tig	1.5
43	R^1 = Oct	R^2 = Oct	11
44			42
45			66
46			440
47			3100

(Table 1) contd….

Cpd			IC$_{50}$(A)/IC$_{50}$(Tg)
48	R^1 = Ac	R^2 = H	2.8
49	R^1 = H	R^2 = Ac	2.5
50	R^1 = Ac	R^2 = Ac	15
37			1.0
35	R^1 = OC$_2$H$_5$	R^2 = H	40
36	R^1 = H	R^2 = OC$_2$H$_5$	2.5

4.2. Targeting Towards Prostate and Prostate Cancer Cells

A prodrug targeted against prostate cancer must fulfill the following requirements:

It must be possible to link the drug used as the starting material to the promoiety part of the prodrug.

After cleavage by the prostate characteristic enzymes a cytotoxic cleavage product (the drug) must be formed.

The prodrug should be selectively cleaved by some of the enzymes characteristic for prostate cancer cells, e.g. PSA or hK2.

The prodrug must be pharmacologically inactive.

The drug must be present in cytotoxic concentrations within the tumor.

The drug should stay in the tumor or be quickly degraded to avoid toxic side effects.
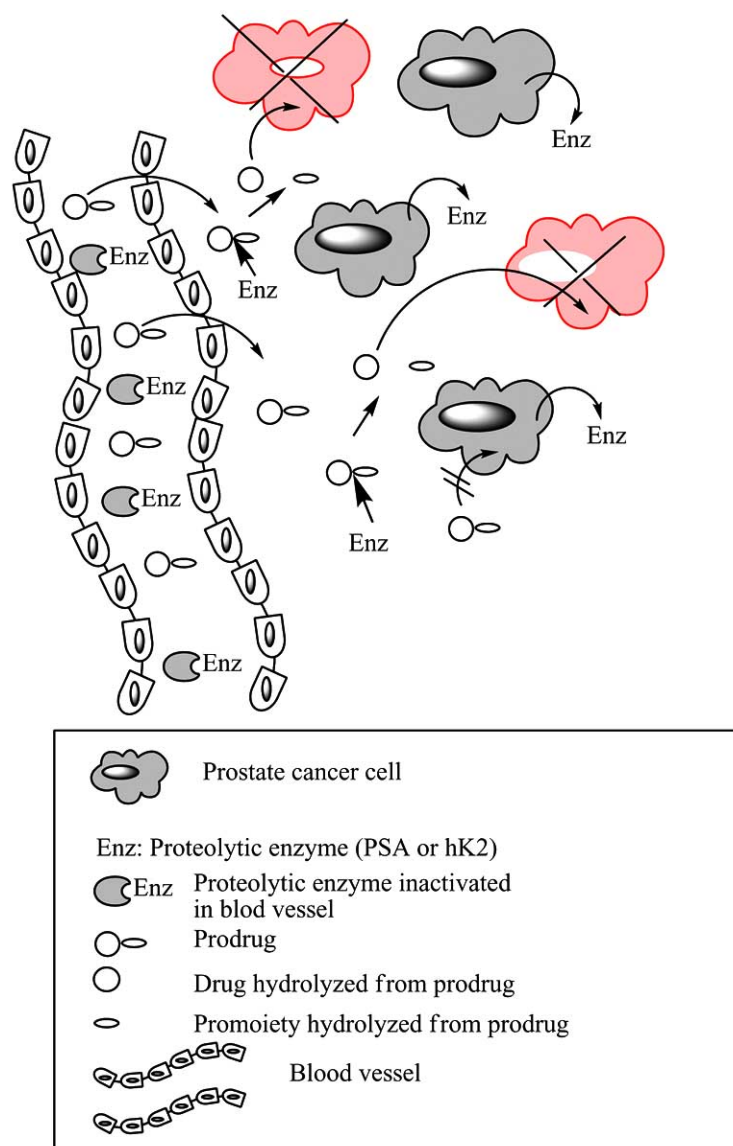
Fig. (12). Targeting of drugs against prostate cancer cells. Prodrugs of drugs acting by penetrating cell membranes and binding to intracellular macromolecules (e.g. the SERCA pump) can be constructed by conjugating the drug to a peptide. Conjugation prevents the drugs from penetrating the cell membrane and thereby neutralizes the cytotoxic effect. By selecting peptides, which are selective substrates for protetolytic enzymes characteristic for the tissue (e.g. PSA or hK2), cleavage will be obtained only in the enzyme secreting tissue to release the cytotoxic drug. A hydrophobic drug will quickly be taken up by nearby cells limiting the cytotoxic effect to this tissue. After systemic administration the prodrug will be distributed in the organism through the blood vessels. Inactivation of the tissue characteristic enzymes (PSA or hK2) by binding to serum proteins prevents cleavage in the vessels.
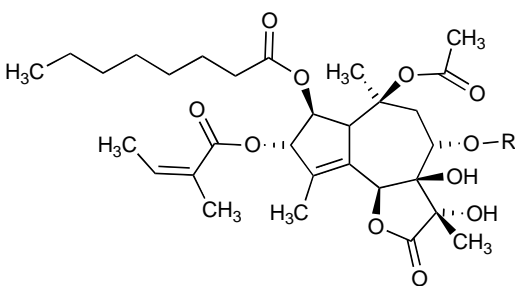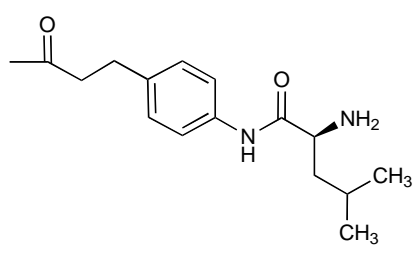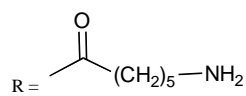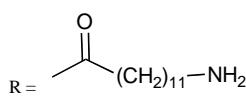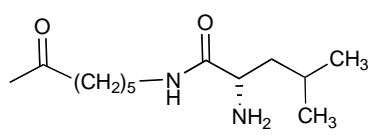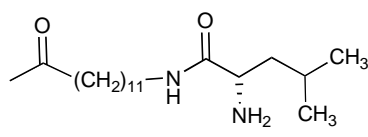
The other cleavage product formed by cleavage of the prodrug should be inert or quickly degraded to inert metabolites.

4.2.1. Feasible Drug

The secretion of proteolytic enzymes from prostate cancer cells makes conjugation of peptide(s) to the drug an attractive strategy for preparation of prodrugs. The absence of amino groups in thapsigargin prevents coupling to the unmodified molecule. The ease of exchanging the acyl group at O8 with a ω-aminoacyl group makes 8-O-ω-aminoacyl esters attractive as target compounds. Based on this strategy a series of compounds were prepared and their SERCA inhibitory activities tested (Table **2**) [29]. Short linkers containing less than 8 methylene groups between the amino group and the carboxylate group were poor inhibitors, whereas prolongation of the linker to more than 10 methylene groups afforded drugs almost equipotent to thapsigargin. Introduction of an aromatic ring in the linker only decreased the biological activity by a minor extent. Conjugation of the amino group with a leucine residue appeared to increase the activity, provided the linker was of sufficient length. The importance of the length of the linker was rationalized when the structure of the Boc derivative of **53** bound to thapsigargin was solved. The structure revealed that the polar amino group pierced the intramembranous helices and appeared on the hydrophilic surface of the SERCA pump if the linker contained a sufficient number of methylene groups, whereas a shorter linker forces the amino group to be inside the pump where it will experience steric and other unfavorable interactions with the SERCA backbone (Fig. (**13**)) [84]. Analogues like **53** and **55** thus are potential drugs for creating a prodrug and fulfilling demands 1 and 2.

Table 2.　　**Potencies as SERCA Inhibitors Or Cytotoxicities of Thapsigargin Analogues Possessing an ω-Aminoacyl Linker at O8**

Cpd		IC$_{50}$(analog) IC$_{50}$(10) Ratios between the concentrations inhibiting SERCA 50%	LC$_{50}$(analog) LC$_{50}$(10) Ratios between the concentrations causing 50% loss of clonogenic survival of TSU-Pr1 cells
51		35	29
52		99	>667
53		2.6	39
54		287	>667
55		3.4	1.0

4.2.2. Prodrugs Activated by PSA

Coupling of the prodrugs to peptides which are substrates for PSA, should afford analogues, from which the cytotoxic drug can be liberated by cleavage of the peptide. At the same time the peptide should only be a substrate for PSA, preventing liberation of the drug outside the prostate tumors. Extensive studies on the substrate specificity of PSA revealed that despite the chymotrypsin like substrate specificity of PSA, substrates containing the amino acid sequence Ser-Lys-Leu-Gln were cleaved on the C-terminal site of the Gln residue [85]. The ability to cleave only on the C-side of Gln may be unique for PSA and suggests some selectivity of PSA for this substrate. Thus, the substrate

His-Cys-Cys-Lys-Leu-Gln-7-amino-4-methylcoumarin

was efficiently cleaved by PSA to give

His-Cys-Cys-Lys-Leu-GlnOH and 7-amino-4-methylcoumarin;

and was found to be inert towards the proteolytic enzymes present in serum [85].

Consequently this peptide was selected as the promoiety part of the prodrug.

Disappointingly, we found that coupling of the peptide to the **53** amine compound (Table **2**) led to a prodrug which was not a substrate for PSA. Attempts to use the same peptide for targeting doxorubicin to prostate cancer cells revealed, that introducing a Leu residue between Gln and doxorubicin afforded a prodrug which was efficiently cleaved by PSA [86]. Application of the same strategy [that is coupling the peptide to **55** (Table **2**)] also was successful. *In vitro* this substrate was cleaved by PSA with a K_m of 475 µM and a k_{cat} of 9.6×10^{-3} s^{-1} to give **55** [19]. As shown in Table **2**, prolongation of the linker with a Leu actually increases the cytotoxicity of the drug. Protection of the terminal amino group with a 4-morpholinocarbonyl group increased stability and solubility of the prodrug affording compound **56** (Fig. **14**) [85].

A prerequisite for the prodrug concept is that the peptide prevents the prodrug from entering the cancer cells and thereby prevents blocking of SERCA. This assumption was verified by incubating non-PSA producing cancer cells (TSU human bladder cancer cells and HCT-116 cells) with thapsigargin, **55** and the prodrug **56**. In both cases thapsigargin and **55** efficiently inhibited the growth whereas **56** did not affect the cells [19]. In contrast PSA producing

cells (LNCaP) were killed by the prodrug **56**. The stability of the prodrug in mouse serum was confirmed by intravenous injections into mice at 7 mg/kg. Only trace amounts of the drug **55** was detected in the serum, whereas the prodrug level reached a peak value of 15 μM and the half-life was estimated to 2.8 h. Finally, the value
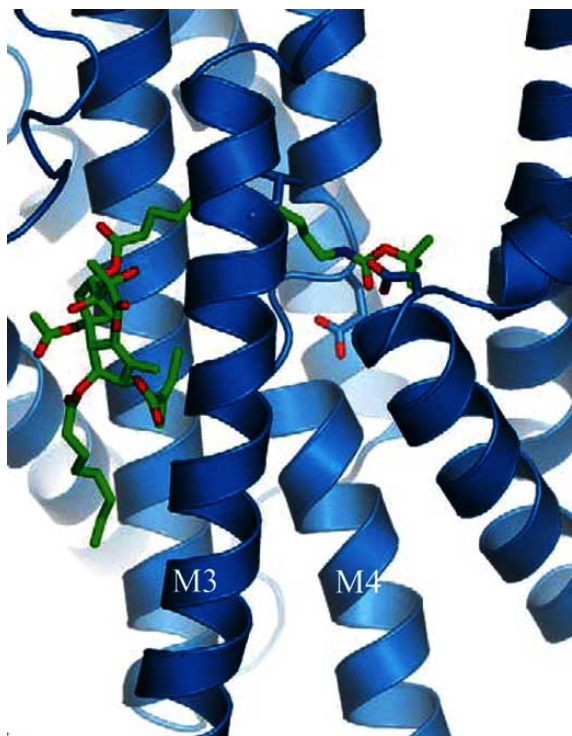

Fig. (13). The localization of the Boc derivative of **53** within SERCA. M1 - M5 indicate first to fifth transmembrane segment, respectively.

of the concept was proven by systemic administration of prodrug **56** to mice inoculated with LNCaP cells and non-PSA producing SN-12C cells. The development of the LNCaP tumors was inhibited, whereas no reduction in the growth of the SN-12C cells could be detected [19].

Prodrugs of doxorubicin [86-88] also have been tested for their ability to control the growth of prostate cancer tumors. In some of these prodrugs the ability of PSA to cleave between Gln and Ser was used as the basis for constructing the promoiety peptide. However, the poor ability of doxorubicin to kill slowly growing prostate cancer cells was reflected by the fact that high concentrations of the drug had to be administered to kill 50% of the tumor cells. As was the case for prodrug **56**, a high selectivity for PSA producing cells was obtained. By systemic administration of a dose near the minimum tolerable dose a reduction in the PSA level and the tumor weight of LNCaP tumors in mice was obtained [87]. A similar approach also has been used for targeting cyclopamine [89], paclitaxel [90], and vinblastine [91] towards PSA producing cells.

The ability of PSA to cleave on the C-side of a tyrosine residue also has been used for making prodrugs that target chloromustards towards PSA-producing cells [92].

4.2.3. Prodrugs Activated by hK2

Studies on large peptide libraries all revealed that efficient cleavage only was obtained on the C-terminal side of arginine residues [80, 93]. Efficient cleavage of peptide substrate were obtained on the C-side of two contiguous arginine residues [93]. Disappointingly the peptide

Ac-Gly-Lys-Ala-Phe-Arg-Arg-Leu

was efficiently hydrolyzed by proteolytic enzymes present in human plasma, apparently preventing selective targeting of prostate cancer cells using hK2 catalyzed cleavage of prodrugs. Fortunately, however, conjugation of this peptide with **55** afforded the prodrug **57** (Fig. **15**), which turned out to be a substrate for hK2 as well as plasmin. hK2 cleaves **57** on the C-side of both arginines affording a
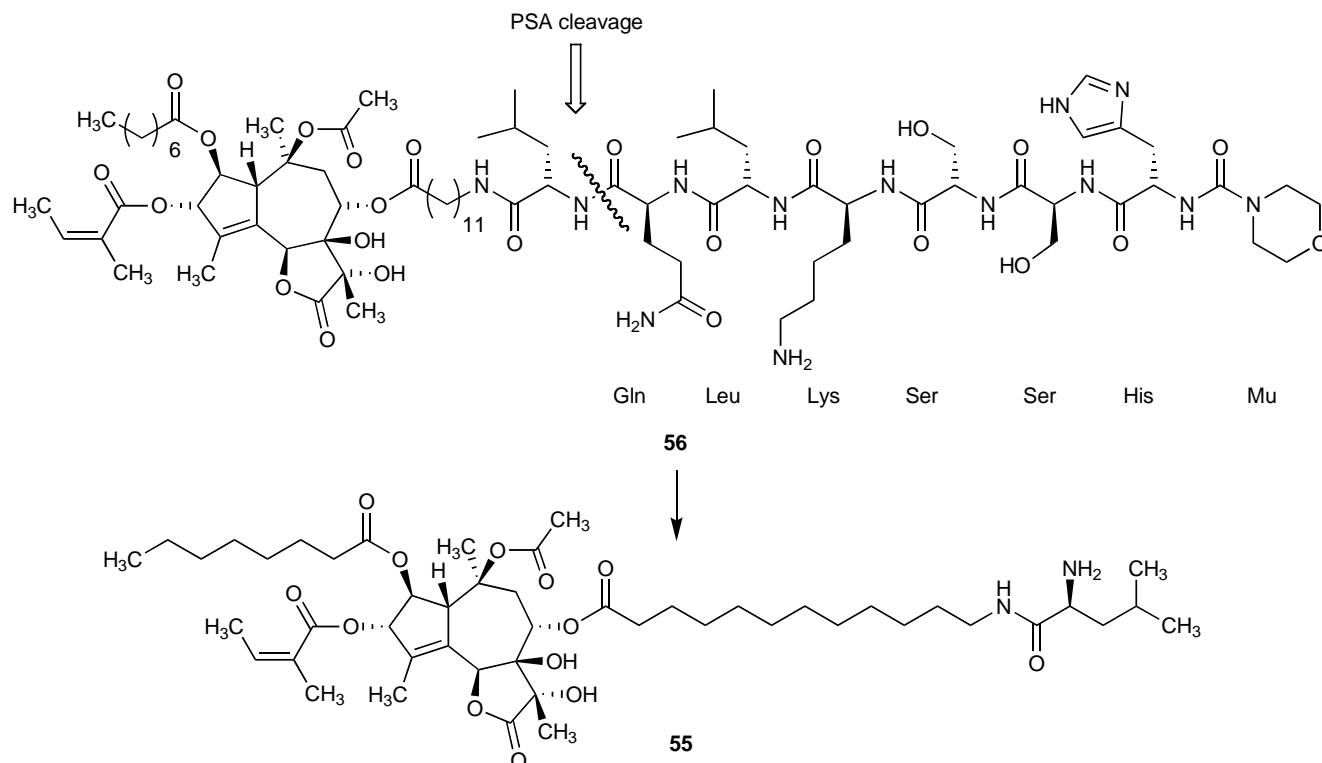

Fig. (14). Absolute configuration of the prodrug **56** and the drug **55** obtained by PSA catalyzed cleavage. The marked bond is sensitive towards PSA.
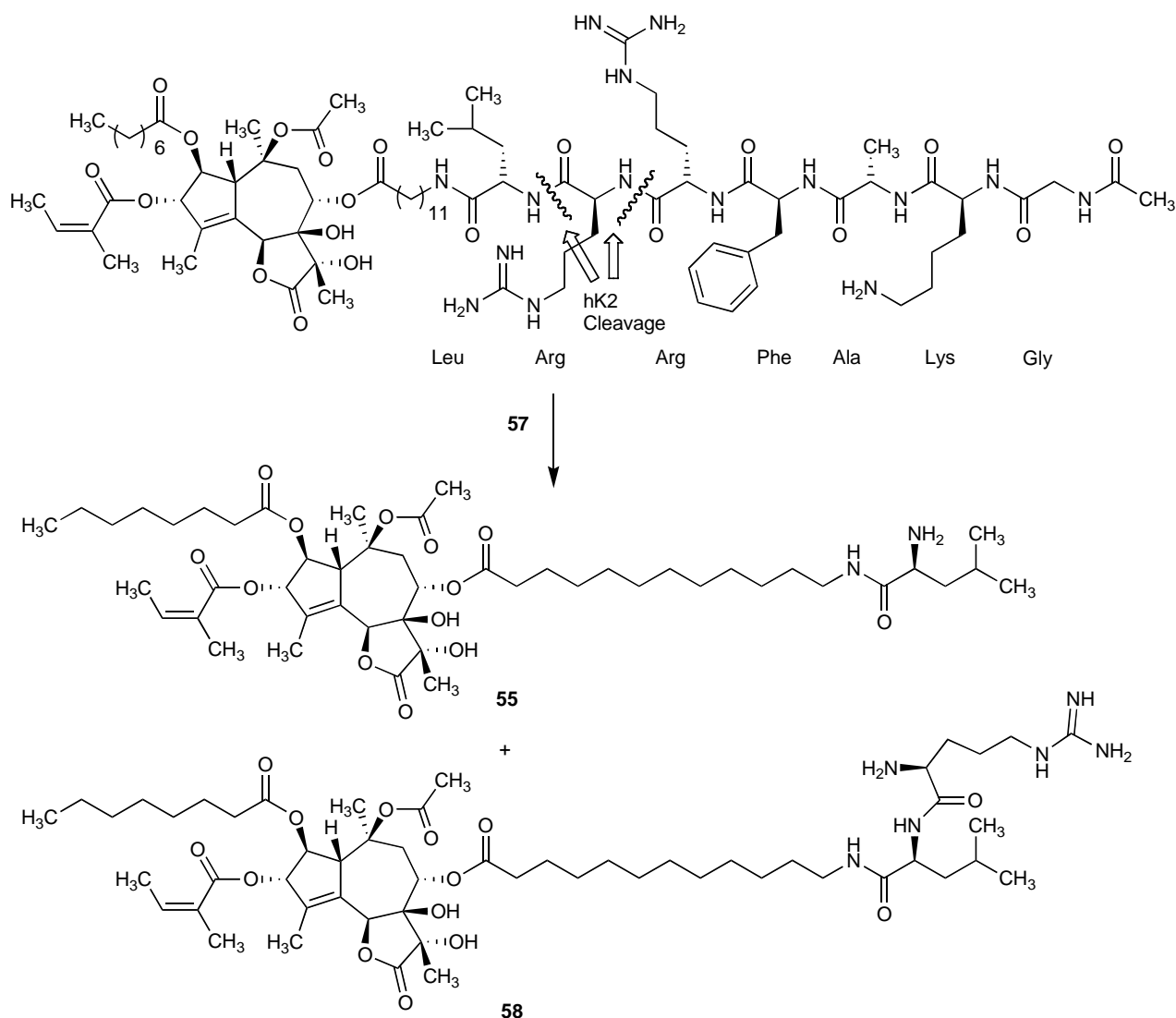
Fig. (15). Prodrug **57** cleaved by hK2 to give **55** and **58**. In serum **57** is protected against cleavage from proteolytic enzymes. The marked bonds are sensitive towards hK2.

2:1 mixture of **55** and **58**. Compound **58** is about 5 times less potent than **55**, probably because of limited ability to penetrate the cell membrane [93]. In spite of this sensitivity to plasmin **57** is not hydrolyzed in either human or mouse serum. This apparently contradictary result can be explained by assuming that **57** is bound to serum proteins making it inaccessible to proteolytic enzymes [93]. Fortunately, these studies suggest that the prodrug might be used for treatment of prostate cancer. An advantage of **57** compared to **56** is the hydrophilic peptide residue in **57** which make the drug much more soluble in water.

Although several approaches for targeting prodrugs against prostate cancer cells taking advantage of PSA secretion are reported in literature only this approach for taking advantage of hK2 has been found.

5. CONCLUSION

The slow growth makes prostate cancer tumors resistant towards available chemotherapeutics, consequently no efficient treatment exists for hormone-refractory prostate cancer, once it has formed metastases outside the prostate gland. Efficient drugs, however, may be developed by targeting the cancer cells with cytotoxins, which kill them in all phases of the cell cycle. In the present publication we describe promising results for selective targeting of prostate cancer cells with prodrugs (**56** and **57**) taking advantage of the high concentration of active prostate specific antigen (PSA) or human glandular kallikrein 2 (hK2) in close vicinity of prostate cells. Cleavage of the prodrug results in liberation of a lipophilic cytotoxin. The lipophilicity causes this agent to be taken up by membranes in close vicinity of where it was formed and thereby limit the distribution to the neighboring cells.

It is expected that an investigational new drug application will be filed within the year for development of drugs based on these principles.

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