Attempts to synthesize a trimeric derivative of pristimerin with potential activity against cancer cells

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ABSTRACT

Pristimerin exhibits significant potential as a therapeutic agent, demonstrating activity against various human cancer cells. The assumption is that the trimer of pristimerin enhances the strength and selectivity of pristimerin-DNA interactions through a multivalent effect. Therefore, to synthesize the trimer of pristimerin, a multi-step route was adopted. The initial step involved the synthesis of 2-(2-(2-azidoetoxy)ethoxy)ethan-1-ol (R1) through an S_N2 reaction, with a yield of 52%. The compound *N*-(*tert*-butoxyl-carbonyl)-tris-(hydroxymethyl)aminomethane (R2), with the amino group protected by *tert*-butoxyl carbamate, was obtained with an 87% yield and, the trialkyne, *N*-(*tert*-butoxyl-carbonyl)-tris-(propargyl)-methyl)aminomethane (R3), was obtained with a 43% yield. Despite successful synthesis of compound R1, R2 and R3, various methodologies were attempted for the transesterification between pristimerin and R1 to produce 2-(2-(2-azidoethoxy)ethoxyethyl pristimerinoate (R4), including chemical and enzymatic hydrolysis of pristimerin as an alternative route. However, none of these attempts succeeded, indicating the remarkable resistance of carbon C-29 in pristimerin to these reactions.

Keywords: *Salacia crassifolia*, Celastraceae, Quinone Methide Triterpene, Transesterification Reaction.

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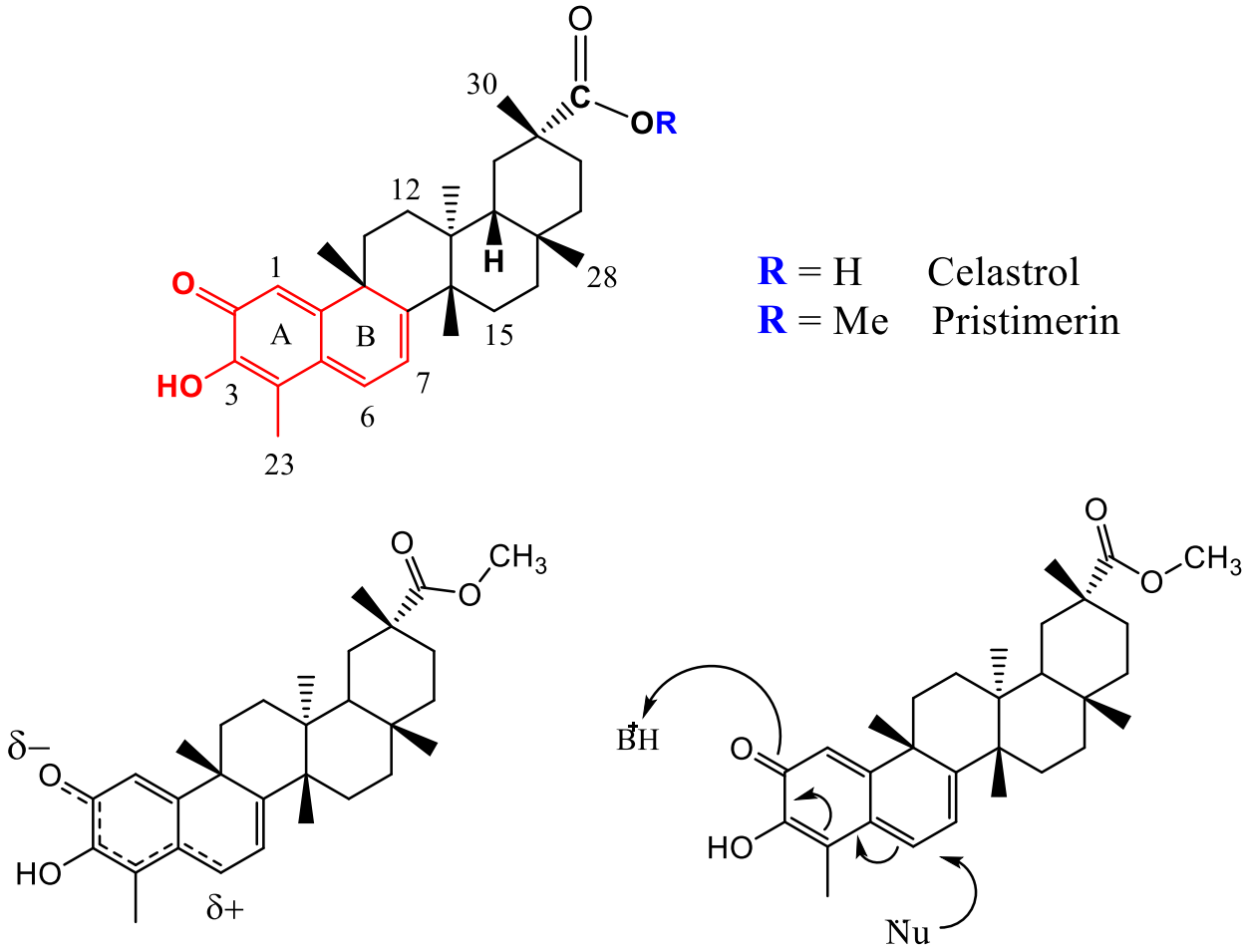
INTRODUCTION

Cancer is a collective term for a group of diseases characterized by the loss of control of cell growth and division, leading to a primary tumor that invades and destroys adjacent tissues and can be followed by metastasis. It is responsible for approximately 14.5% of all deaths worldwide, with its incidence increasing due to the aging of the population in most countries (Avendaño and Menéndez, 2015; Kubczak et al., 2021). Despite the rapid evolution of diagnostic methods and therapies, metastasis and resistance to administered drugs are the main obstacles to treatment success. One of the treatment alternatives involves the use of chemotherapy (Huang et al., 2016; Kubczak et al., 2021). In this context, chemical substances obtained from natural sources represents a promisor alternative to obtain new anti-cancer compounds (Huang et al., 2021). They can exhibit similar anti-cancer potential, usually with reduced side effects. It was reported that natural compounds obtained from plants, *e.g.,* polyphenols, flavonoids, stilbenes, carotenoids and acetogenins, might be effective against cancer cells *in vitro* and *in vivo* (Huang et al., 2016; Kubczak et al., 2021). Several published results indicate the activity of natural compounds on protein expression by its influence on transcription factors. They could also be involved in alterations in cellular response, cell signaling and epigenetic modifications (Huang et al., 2016; Huang et al., 2021; Kubczak et al., 2021). Among the natural compounds currently being studied, pristimerin stands out.

Pristimerin is a quinone methide triterpene considered as one of the taxonomic markers of species of the family Celastraceae, commonly isolated from its roots. Quinone methides consist of five six-membered rings, with one carbonyl group on the carbon C-2, one hydroxyl group on the carbon C-3 and an unsaturated system involving the rings A and B (Corsino et al., 2000). Besides that, the carbon C-6 of such compounds display an electrophilic character. The earliest compounds described for this class are celastrol and pristimerin (Figure 1), which have been isolated from *Tripterygium wilfordii* Hook, *Celastrus scandens* L. and *Pristimera indica* (Gunatilaka, 1996). And, in 2020, Dos Santos and collaborators isolated pristimerin from hexane/ethyl ether extract obtained from roots of *Salacia crassifolia* in a significant amount (5 g), constituting the main compound of this extract (10%) (Dos Santos et al., 2020).

Since its isolation in 1951 and structural elucidation in 1954, different biological activities have been attributed to pristimerin (Kulkarni and Shah, 1954). Among these activities are anti-inflammatory (Kim, Park and Kim, 2013; Zhao et al., 2020), antioxidant (Shaaban et al., 2018), antimalarial (Figueiredo et al., 1998), antibacterial (López, de León and Moujir, 2011), and anti-tumor (Yousef et al., 2016; Yousef et al., 2017; Lee et al., 2018; Cevatemre, et al., 2018; Zhao et al., 2019; Li et al., 2019; Chen et al., 2021; Al-Tamimi et al., 2022).

Figure 1: Chemical structure of celastrol and pristimerin and electrophilic properties of pristimerin.



Source: Dos Santos et al., 2024.

The cytotoxic activity observed in several tumor lines was related to the fact that pristimerin presents antiproliferative activity involving apoptosis or programmed cell death (Li et al., 2019), autophagy (Cevatemre, et al., 2018), and inhibition of signaling pathways such as nuclear factor Kappa B (NF-κB) (Tu et al., 2018), Akt (kinase protein B, PKB) (Park and Kim, 2018), modulation of MAPK signaling pathways (Al-Tamimi et al., 2022), among others. It was observed that the use of pristimerin together with taxol (paclitaxel), a tetracyclic diterpene isolated from bark of *Taxus brevifolia* Nutt. (Family Taxaceae), induces the autophagy process in human breast cancer cells through the regulation ERK1/2 (Lee et al., 2018). A nano-delivery system constituted by folic acid-modified nano-herb micelle was developed for codelivery of pristimerin and paclitaxel to enhance chemosensitivity of malignant neoplasms, such as non-small cell lung cancer (NSCLC), in which pristimerin could synergistically enhance paclitaxel-induced growth inhibition of A549 cancer cell (Chen et al., 2022).

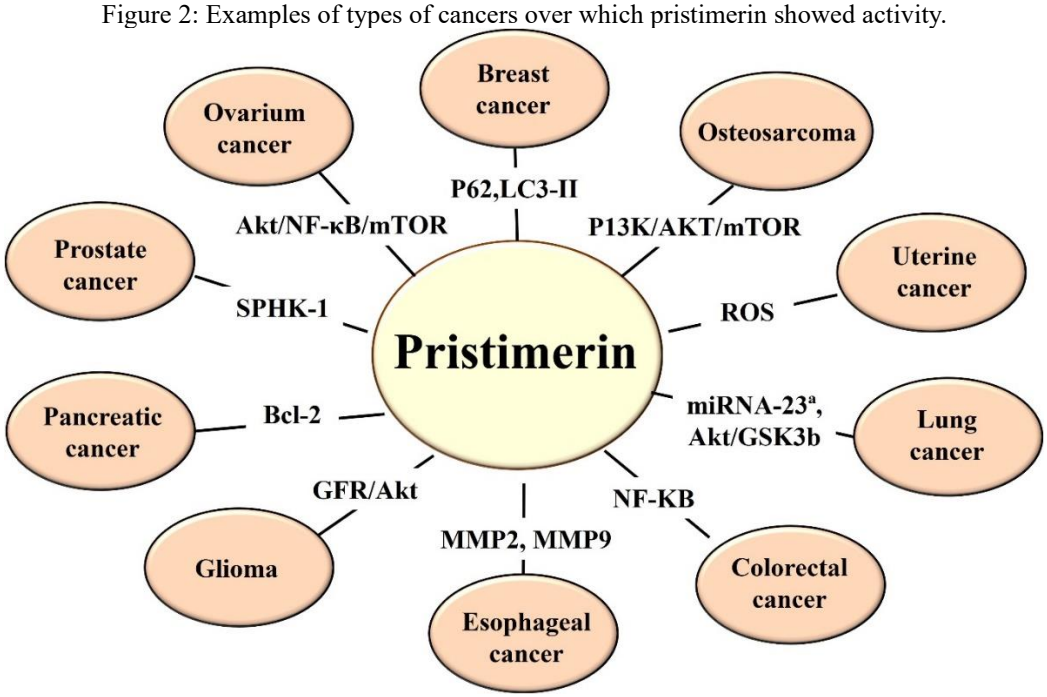
It is already known that, in general, quinone methides react with the nitrogenous bases of DNA by alkylation. The electrophilicity of carbon 6 of pristimerin may account for its property as an anti-tumor since allows its reaction as an alkyl group donor in DNA alkylation reactions (Figure 1). It

is known that these reactions can lead to the depurination of that nucleic acid and, consequently, to cell unfeasibility (Yang et al., 2008; Toteva and Richard, 2011). Thus, it is possible that pristimerin have anti-tumor activity because it is capable to alkylating the DNA of tumor cells.

Alkylating agents can be defined as compounds capable of covalently attaching an alkyl group to a biomolecule under physiological conditions (Hall and Tilby, 1992). DNA alkylating agents interact with resting and proliferating cells at any stage of the cell cycle, being more cytotoxic during the late G1 and S phases. For more than sixty years, alkylating agents have been used for the treatment of cancer and new agents in this class of drugs have been developed (Hall and Tilby, 1992; Lossos et al., 2019). These agents act during all phases of the cell cycle, directly in the DNA, generally alkylating the residues of *N*-7-guanine, leading to the rupture of the DNA filaments, which results in an abnormal base pairing, inhibition of cell division and cell death (Lossos et al., 2019).

Pristimerin is an alkylating agent that covalently binds to DNA, inducing apoptosis by different signalling pathways in human cancer cell lines in the pancreas (Deeb et al., 2014), glia (Yan et al., 2013), prostate (Liu et al., 2015), colon/rectum (Yousef et al., 2016), melanoma (Zhang et al., 2017), breast (Xie et al., 2016; Lee et al., 2018; Zhao et al., 2019), skin (Al-Tamimi et al., 2022), oesophagus (Huang et al., 2019), oral cavity (Wu et al., 2019), lung (Zhang et al., 2019), and ovarium (Gao et al., 2014) (Figure 2).

Figure 2: Examples of types of cancers over which pristimerin showed activity.



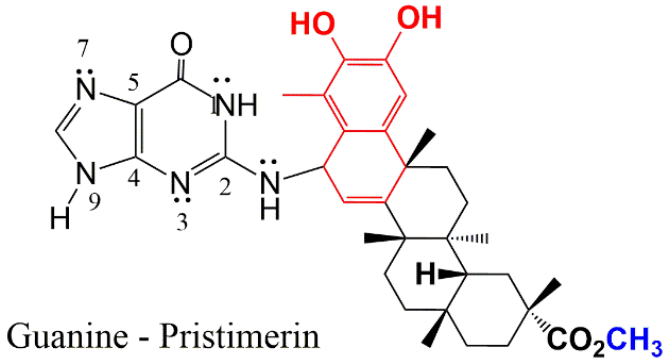
Source: Dos Santos et al., 2024.

Due to the promising pharmacological effects observed, the cellular and molecular mechanisms of action of quinone methide pristimerin have been studied in cancer therapy (Li et al., 2019; Chen et al., 2021). Quinone methides are Michael's acceptors, therefore they are electrophilic

compounds capable of promoting DNA alkylation and reticulation (Wang et al., 2017; Minard et al., 2019). However, the mutagenic potential of these alkylating agents is not yet fully investigated.

The formation of the quinone methide adduct with the nitrogenous base of the DNA can contribute to the elucidation of the mechanism of alkylation of this nucleic acid and serves as a model for the development of new quinone methides with alkylating property and potential anti-tumoral activity. Thus, based on the results found by Zhou *et al* (2011), it is assumed that pristimerin also forms an adduct with guanine, contributing to establish a possible mechanism that explains the high anti-cancer potential of pristimerin (Figure 3).

Figure 3: Possible adduct that would be formed between guanine and pristimerin.



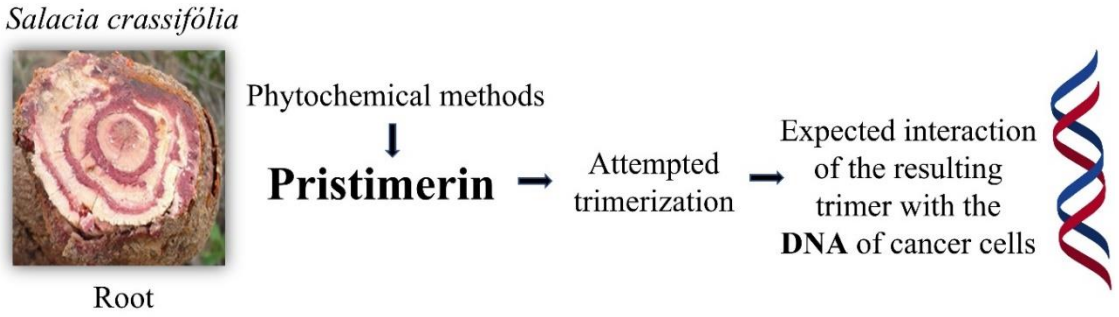
Source: (Dos Santos et al., 2024).

In this context, a trimer of pristimerin can cause an increase in the strength and selectivity of pristimerin-DNA interactions through a multivalence effect. Multivalence is involved in a great diversity of biological interactions, due to the multiple interactions existing between ligand pairs and specific receptors. The use of multivalent interactions represents a good tool for obtaining supramolecular compounds, which allows the formation of a strong and reversible covalent bond, significantly increasing the bond selectivity when compared to monovalent bonds (Dubacheva et al., 2017; Myung et al., 2018).

In view of the above and the significant amount of isolated pristimerin from *Salacia crassifolia* G.Don., a plant of the family Celastraceae (Dos Santos et al., 2020), the synthetic route shown in Figure 5 was proposed to obtain a trimer of pristimerin. The units of pristimerin with azidic oligoethyleneglycol spacer would be introduced in the branched trialkyne by means of click chemistry. Later, it was intended to investigate the possible formation of the adduct with nitrogenous bases of the DNA. For this purpose, the pristimerin trimer would be connected to a proper resin and the ability of alkylation would be evaluated in the presence of guanine, for example.

Thus, the objective of the present work was to obtain a trimer of pristimerin previously isolated by Dos Santos et al., (2020), in order to evaluate the formation of adduct with a DNA nitrogen base of cancer cells (Figure 4).

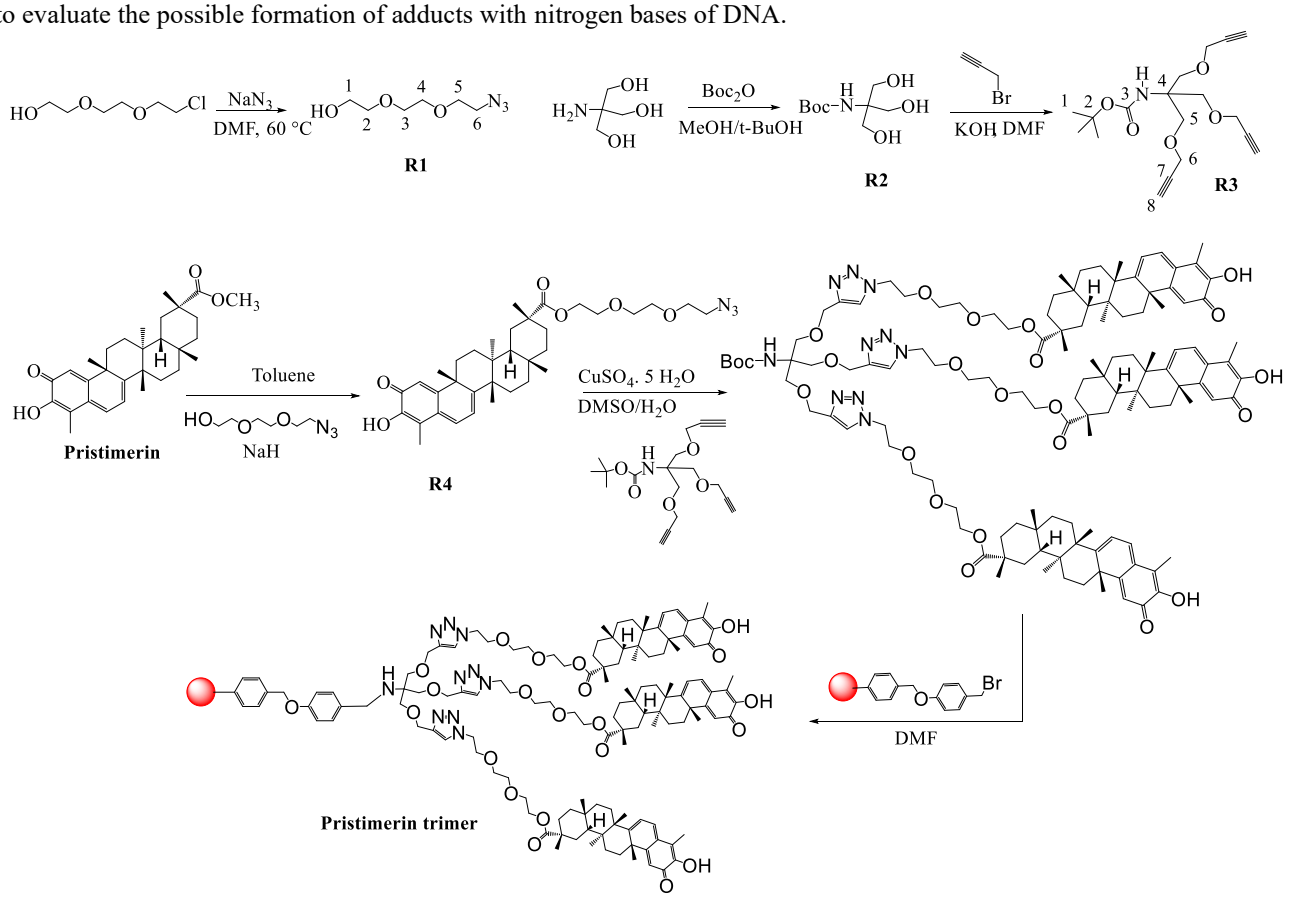
Figure 4: Summary of obtaining the trimer of pristimerin from roots of *Salacia crassifolia* aiming its interaction with the DNA of cancer cells.



Source: Authors own.

To achieve this objective, the following steps were planned to synthesize: a) the alcohol 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol (**R1**), b) the *N*-(*tert*-butoxyl-carbonyl)-tris-(hydroxymethyl)-aminomethane (**R2**), c) the *N*-(*tert*-butoxyl-carbonyl)-tris-((propargyl)methyl)-aminomethane (**R3**), d) the 2-(2-(2-azidoethoxy)ethoxyethyl pristimerinoate (**R4**) and e) perform a click chemistry reaction between **R3** and **R4** (Figure 5).

Figure 5: Synthetic route proposed to obtain the pristimerin trimer. The red ball corresponds to a connection with a resin to evaluate the possible formation of adducts with nitrogen bases of DNA.



Source: (Dos Santos et al., 2024).

MATERIAL AND METHODS

GENERAL EXPERIMENTAL PROCEDURES

Thin-layer chromatography (TLC) processes were performed with silica gel 60 G (7 g/15 mL water) on a 0.25 mm thick glass plate previously activated at 100 ºC. The chromatographic plates were revealed by spraying solution (1:1) of 3% perchloric acid in water with 1% vanillin in ethanol (Court and Iwu, 1980; Wagner and Bladt, 1996). Column chromatography (CC) was performed with silica gel 60, using an appropriate eluent. Compounds with adequate purity were submitted to IR and ^{1}H and ^{13}C NMR spectrometry. The melting points of the compounds were determined using the digital MQAPF-302 equipment from Microquímica Equipamentos Ltda. The infrared spectra of compounds, as ~1% KBr pellets, were recorded on a Shimadzu IR-spectrometer IR-408. The ^{1}H and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, using a Brüker DRX400 AVANCE spectrometer and $CDCl_3$ or DMSO were used as solvent. The chemical shift assignments were registered in ppm (δ) using tetramethylsilane (TMS) as the internal reference ($\delta_H = \delta_c = 0$), and the coupling constants (J) were registered in Hertz (Hz) (Dos Santos et al., 2024).

SYNTHESIS PROCEDURES

The synthesis of 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol **(R1)** (Figure 5) was carried out according to Wallat *et al*. (2017). In a 125 mL reaction flask, 5.2 g of sodium azide (29.7 mmol), 15 mL of *N,N*-dimethylformamide (DMF) and 2-(2-(2-chloroethoxy)ethoxy)ethanol (862 µL, 5.9 mmol) were added. The mixture was maintained at 60 °C, with constant magnetic stirring. After 48 hours, the azide was filtered and 30 mL of water-dichloromethane 1:1 solution was added to the mixture. Then, the mixture was transferred to a separation funnel and the organic phase was washed (2 x 15 mL) with distilled water. After drying with anhydrous Na_2SO_4 and filtration, 15 mL of ethanol were added, and the solvent was removed in a rotary evaporator. The residue obtained was purified through silica gel CC eluted with ethyl acetate. The product **R1** was isolated with 53 % yield (555.0 mg).

R1 (2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol): Molecular formula: $C_6H_{13}N_3O_3$, Molar mass: 175.1 g mol^{-1}. Appearance: yellow oil. **^{1}H NMR** (400 MHz, $CDCl_3$, δ_H (ppm), multiplicity, integration, *J* (Hz), attribution: 2.0 (s, 1 H, H-6); 2.53 (s, 1H, H-6), 3.41 (t, 2 H, *J*=5.1 Hz, H-5), 3.62 (dd, 2 H, *J*=5.4 and 4.5 Hz, H-2), 3.68 (s, 4H, H-3 and H-4) and 3.74 (t, 2 H, *J*= 4.5 Hz, H-1). **^{13}C NMR** (100 MHz, $CDCl_3$): δ_C (ppm): 50.71 (CH_2, C-6); 61.74 (CH_2, C-1); 70.03 (CH_2, C-4); 70.39 (CH_2, C-2); 70.69 (CH_2, C-5) and 72.60 (CH_2, C-3) (Dos Santos et al., 2024).

The synthesis of *N*-(*tert*-butoxyl-carbonyl)-tris-(hydroxymethyl)-aminomethane **(R2)** (Figure 5) was performed in accordance to Segura *et al*., (2001). In a 50 mL reaction flask, 1g of tris(hydroxymethyl)-aminomethane was added to a MeOH:*t*-BuOH (1:1) mixture. A solution of di-

tert-butoxyl dicarbonate dissolved in *t*-BuOH was then dripped into the mixture. Until the end of the addition of this solution, the reaction mixture was kept at low temperature, using an ice bath. After 20 hours of reaction the solvent was removed using a rotary evaporator. To the obtained residue was added cooled ethyl acetate, which induced the formation of a white solid, which was vacuum filtered leading to the obtaining of the **R2** with 87 % yield (1.58 g).

R2 (*N*-(*tert*-butoxyl-carbonyl)-tris-(hydroxymethyl)aminomethane)**:** Molecular formula: $C_9H_{19}NO_5$. Molar mass: 221.25 g mol^{-1}. Appearance: White solid. **^{1}H NMR** (400 MHz, DMSO, δ_H (ppm), multiplicity, integration, *J* (Hz), attribution: 1.37 (s, 9H, CH_3, H-1); 3.52 (s, 6H, CH_2, H-5) and 5.77 (s, 1H, NH). **^{13}C NMR** (100 MHz, DMSO): δ_C (ppm): 28.23 (CH_3, C-1); 60.25 (CH_2, C-5); 60.42 (C); 77.84 (C-2) and 155.03 (C-3) (Dos Santos et al., 2024).

The synthesis of *N*-(*tert*-butoxyl-carbonyl)-tris-((propargyl)-methyl)aminomethane (**R3**) (Figure 5) was performed in accordance to Segura *et al*., (2001). In a 100 mL round-bottom flask, cooled in an ice bath, 0.5 g of **R2** dissolved in 6 mL of DMF, and 1.46 mL of propargyl bromide (80 % in toluene) were added. KOH powder (951 mg) was added in small portions over 15 minutes. The reaction was kept under inert argon atmosphere at 35 ºC for 24 hours. After this time, 10 mL of ethyl acetate (AcOEt) were added. The mixture was transferred to a separation funnel, extracted with 40 mL of AcOEt and washed with distilled water (3 x 30 mL). The organic phase was dried with anhydrous Na_2SO_4, filtered and the solvent was removed in a rotary evaporator. The residue was purified by silica gel CC using a hexane-AcOEt (9:1) mixture as the eluent. The compound **R3** was isolated with 42 % yield (315.0 mg).

R3 (*N*-(*tert*-butoxyl-carbonyl)-tris-((propargyl)methyl)-aminomethane): Molecular formula: $C_{18}H_{25}NO_5$. Molar mass: 335.40 g mol^{-1}. Appearance: yellowish oil. **^{1}H NMR** (400 MHz, $CDCl_3$, δ_H (ppm), multiplicity, integration, *J* (Hz), attribution: 1.43 (s, 9H, H-1), 2.42 (t, 3H, *J* = 2,4 Hz, H-8), 3.79 (s, 6H, H-5), 4.16 (d, 6H, *J*= 2.4 Hz, H-6) and 4.93 (s, 1H). **^{13}C NMNR** (100 MHz, $CDCl_3$): δ_C (ppm): 28.35 (CH_3, C-1), 58.09 (CH_2, C-6), 58.67 (C, C-2), 68.93 (CH_2, C-5), 74.56 (CH, C-8), 79.63 (C, C-7) and 154.79 (C, C-3) (Dos Santos et al., 2024).

To synthesize the derivative **R4** (2-(2-azidoethoxy)ethoxyethyl pristimerinoate) (Figure 5) through transesterification, we initially followed the method outlined by Koval et al. (2008), and subsequently adopted the approach described by Chavan et al. (2004).

For the first transesterification attempt, inspired by the method of Koval et al. (2008), a Soxhlet apparatus filled with 4A molecular sieve and anhydrous toluene, was employed. In a 100 mL two-neck round-bottom flask, 100 mg of pristimerin with 20 mL of anhydrous toluene were added. In this solution a pre-prepared mixture containing 15 mg of NaH, 10 mL of anhydrous toluene and 49 mg of **R1** were slowly added. The reaction was maintained at 100 ºC and after 24 hours was processed. The reaction was monitored by TLC and the desired compound was not obtained.

Subsequently, we attempted transesterification following the procedure outlined by Chavan et al. (2004). In a 50 mL round-bottom flask attached to a reflux condenser, were added pristimerin (100 mg), **R1** (44 mg), iodine (8 mg) and 10 mL of toluene. The reaction was maintained at 115-120 ºC in a glycerin bath, for 4 hours. After this period, it was observed by TLC that the starting material was still in the reaction medium. The reaction condition was then maintained for another 4 hours, but still no significant change was observed. After cooling, the mixture was washed with a $Na_2S_2O_3$ solution and then with a saturated NaCl solution. The organic phase was dried with anhydrous Na_2SO_4, filtered and the solvent removed in a rotary evaporator. TLC was performed and it was observed that no product was formed.

Therefore, an alternative to obtain **R4** was to attempt the hydrolysis of pristimerin to obtain celastrol (Figure 1) and subsequent esterification of this compound with **R1**. The hydrolysis reaction used to obtain celastrol from pristimerin was performed using the method reported by Camelio, Johnson and Siegel (2015). In a 100 mL round-bottom flask, 200 mg of pristimerin, 75 mL of 1,4-dioxan:water (1:1) and 450 mg of KOH were added. The reaction was maintained at 100 ºC for 4 hours in an oil bath. After slow cooling until room temperature was reached, the reaction medium was then acidified with phosphate buffer (pH 2). After the addition of 100 mL of AcOEt, the mixture was transferred to a separation funnel, and 150 mL of phosphate buffer (pH 2) were added. The organic phase was dried with anhydrous Na_2SO_4, filtered and the solvent removed in a rotary evaporator. TLC was performed and it was observed that there was no formation of the expected celastrol.

Another attempt to obtain celastrol was performed using enzymatic hydrolysis methods adapted from that as described by Ciuffreda, Casati and Santaniello (2000). The enzymes tested were: Lipases A and B from *Candida antarctica* (CAL-A and CAL-B, respectively); lipase of *Burkholderia cepacea*, (PCL); lipase of *Rhizomucor miehei* (RMIM) the protease alpha-chymotrypsin. All lipases were used as commercial immobilized biocatalysts, whereas alpha-chymotrypsin was used as a crude extract. Methods **A** to **E** were tested.

Method A: In a 10 mL round-bottom flask, 50 mg of pristimerin, 3.60 mL of *tert*-butoxyl-methyl ether, 120 µL of distilled water and 5 mg of immobilized lipase (PCL, RMIM, CAL-A or CAL-B) were added. The reaction was performed at room temperature under gentle magnetic stirring. The reaction was monitored by TLC and no product was observed within 72 hours after the start of the reaction. New attempts were conducted under analogous conditions, with variations introduced in the choice of solvent. Using 3.0 mL of *tert*-butoxyl methyl ether and 600 µL of distilled water, there was no formation of the product celastrol. For the enzyme CAL-B the procedure was also performed using toluene instead of *tert*-butoxyl methyl ether. No formation of celastrol was observed within 3 hours after the beginning of the reaction.

Method B: In a 10 mL round-bottom flask containing 50 mg of pristimerin, 1.4 mL of *tert*-butoxyl methyl ether, 3.6 mL of sodium phosphate buffer solution (0.1 M, pH 7.2) and 4 mg of CAL-B were added. The reaction was maintained at 30 ºC, under constant stirring on an orbital stirring table (300 rpm) for 72 hours. Then, HCl was added until reaching pH 3-4, and the mixture was submitted to extraction with ethyl acetate (2 x 5 mL). TLC analysis of both phases did not reveal celastrol formation.

Method C: In a 10 mL round-bottom flask, 50 mg of pristimerin, 2.3 mL of THF and 0.1 mL of sodium phosphate buffer (0.1 M, pH 7.2) and 12.5 mg of alpha-chymotrypsin were added. The mixture was kept at 38 °C under stirring (300 rpm) for 72 hours. Then, the aqueous phase was treated with HCl up to pH 3-4 and extracted with ethyl acetate (2 x 4.0 mL). There was no formation of the expected celastrol.

Method D: In a 10 mL round-bottom flask, 50 mg of pristimerin were added; 2.3 mL of toluene, 0.1 mL of water and 9 mg of CAL-B. The reaction was maintained at 70 °C under magnetic stirring for 72 hours. The extraction was performed with ethyl acetate (2 x 4.0 mL). And the aqueous phase was acidified until pH 3-4, then it was extracted using ethyl acetate (2 x 4.0 mL). Analysis by TLC indicated that there was no formation of the celastrol.

Method E: In a 10 mL round-bottom flask, 50 mg of pristimerin, 5.0 mL of sodium phosphate buffer (0.1 M, pH 7.5) and 12.5 mg of chymotrypsin were added. The reaction was maintained at 38 °C under agitation (300 rpm). After 72 hours, the reaction was extracted with ethyl acetate (2 x 4.0 mL) and the aqueous phase was acidified at pH 3-4. Then it was extracted with ethyl acetate (2 x 4.0 mL). By TLC it was observed that there was no formation of the celastrol.

RESULTS AND DISCUSSION

Pristimerin is the major metabolite isolated from hexane/ethyl ether extract of *Salacia crassifolia* roots, being obtained 5 g, equivalent to 10% of the crude extract (Dos Santos et al., 2020). Due to this availability, the objective of this work was to obtain a pristimerin trimer, from the synthesis route presented at Figure 5, using the derivatives **R1** to **R4,** and prove that this trimer would form adduct with nitrogenous bases of DNA by alkylation.

Compound 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol (**R1**) was obtained by means of bimolecular nucleophilic substitution (S_N2) reaction. The reaction was monitored by TLC and after 48 hours it was observed that the starting material had already been consumed. After elaboration and purification by CC the 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol (**R1**) was obtained with 53 % yield, as a light yellow oil soluble in chloroform with Rf smaller than the starting material. The structure of **R1** has been confirmed through IR and NMR spectral data. In the IR (KBr, cm^{-1}) spectrum of **R1**, the absorption band at 2110 cm^{-1} is characteristic of stretching of the azide group and the broadband at

3423 cm^{-1} was attributed to the O-H stretching of alcoholic hydroxyl group. In the ^{1}H NMR spectrum (400 MHz, $CDCl_3$), two triplets were observed at δ_H 3.41 (t, 2H, *J*=5.1 Hz, H-5) and at δ_H 3.74 (t, 2H, *J*=4.5 Hz, H-1). Two large singlets were observed at δ_H 2.00 (sl, 1H, H-6) and 2.53 (sl, 1H, H-6) and were ascribed to the most shielded hydrogen atoms, which are bonded to the carbon atom bearing the azide group. In the ^{13}C NMR and DEPT-135 spectra (100 MHz, $CDCl_3$), six signals ascribed to the methylene carbons were observed in δ_C 50.71 (C-6), 61.74 (C-1), 70.03 (C-5), 70.39 (C-4), 70.69 (C-2) and 72.60 (C-3), confirming the **R1** structure (Dos Santos et al., 2024).

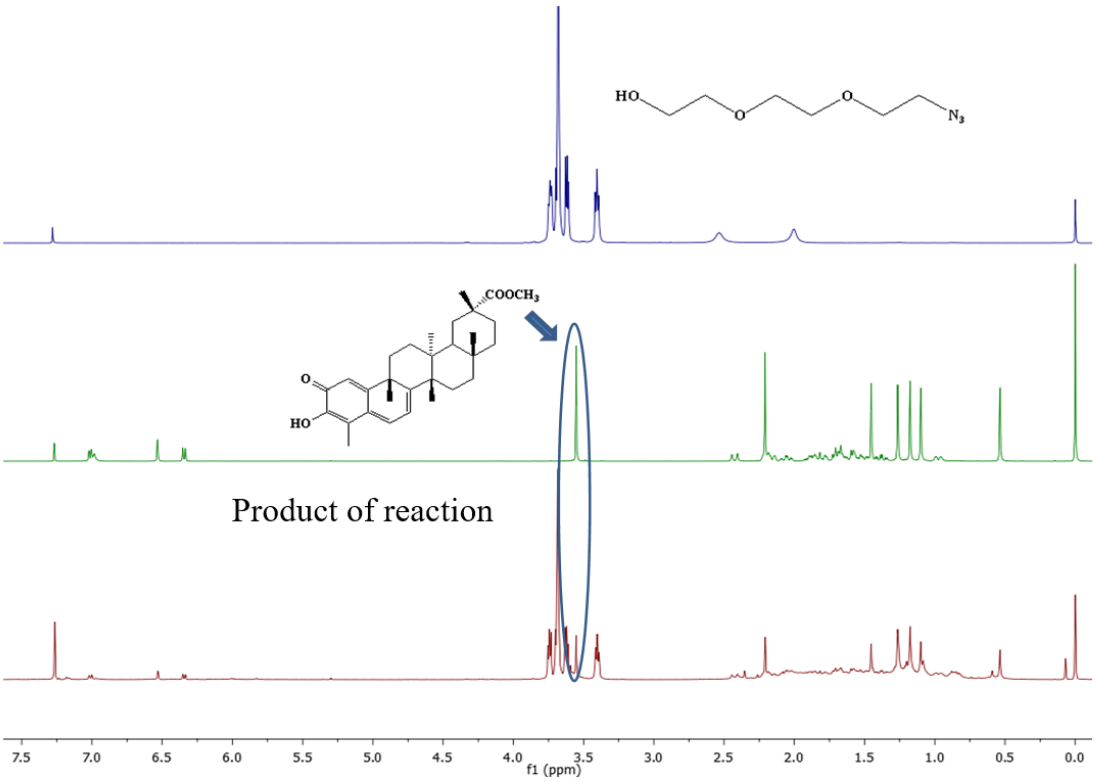
The protection of the amino group of **R1** with *tert*-butoxyl carbamate (Boc) led to compound *N*-(*tert*-butoxyl-carbonyl)-tris-(hydroxymethyl)-aminomethane (**R2**) as a white solid, with a yield of 87 % and melting point 144-149 ºC. The IR spectrum (KBr, cm^{-1}) of **R2** showed an absorption band at 3306 cm^{-1}, characteristic of hydroxyl stretches and also at 1679 cm^{-1} corresponding to the C=O bond stretch of carbamate carbonyl. The ^{1}H NMR spectrum (400 MHz, DMSO) of **R2** showed a singlet at δ_H 1.37 (s, 9H, CH_3, H-1) ascribed to the hydrogen atoms of the *tert*-butoxyl group of Boc. The singlet at δ_H 3.52 (6H) was attributed to hydrogen atoms of methylenic carbon (H-5). The broad signal observed at δ_H 5.77 was assigned to the hydrogen the N-H. The signal at δ_C 28.23 in the ^{13}C NMR spectrum (100 MHz, DMSO) of **R2** was attributed to methyl carbon (C-1). The carbon signals at δ_C 60.25 and δ_C 60.42 were attributed to the three methylenic carbon atoms (C-5) and the signals at δ_C 77.84 (C-2) and at 155.03 (C-3) were assigned to non-hydrogenated carbon atoms (Dos Santos et al., 2024).

The compound *N*-(*tert*-butoxyl-carbonyl)-tris-((propargyl)methyl)-aminomethane (**R3**) was obtained by means of the S_N2 reaction between **R2** and propargyl bromide. The reaction was monitored by TLC, in which a well-defined stain was observed after revelation with $KMnO_4$ solution indicating the presence of the alkyne group. After purification, using CC silica gel, the product **R3** was obtained as a yellow oil with 42% yield. The IR (KBr, cm^{-1}) spectrum of **R3** showed absorption bands at 1715 cm^{-1}, corresponding to carbonyl stretch, at 2118 cm^{-1} characteristic of stretching C≡C bond and at 3294 cm^{-1} attributed to the C-H stretch of terminal alkyne. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of **R3** showed a triplet at δ_H 2.40 (t, 3H, *J*=2.4 Hz) associated with the alkyne terminal hydrogen atom (H-8) and a duplet at δ_H 4.16 (d, 6H, *J*=2.4 Hz) assigned to the methylenic hydrogen (H-6) atoms of the propargylic chain. The ^{13}C and DEPT-135 NMR (100 MHz, $CDCl_3$) spectra allowed to attribute the signals at δ_C 74.56 (CH) to the carbon C-8 and the signals at δ_C 79.63 (C-7) and δ_C 154.79 (C-3) to carbon non-hydrogenated. The signal at δ_C 58.09 (C-6) was attributed to the methylenic carbon atoms of the propargylic chain (Dos Santos et al., 2024).

In relation to the transesterification of pristimerin aiming to obtain 2-(2-(2-azidoethoxy)ethoxyethyl pristimerinoate (**R4**), two attempts of reaction were carried out. The first attempt was an adaptation of the transesterification method described by Koval *et al*., (2008). The

reaction carried out at 100 °C under stirring and monitored by TLC. However, the starting material had not been consumed after 24 h. Based on the ^{1}H NMR spectrum (Figure 6) of the material obtained after reaction elaboration process it was concluded that there was no formation of the desired product.

Figure 6: ^{1}H NMR spectra (400 MHz, $CDCl_3$) of R1 spectra (400 MHz, $CDCl_3$) of R1 (Top); pristimerin (middle) and of the product of the reaction (below).



Source: (Dos Santos et al., 2024).

The singlet at δ_H 3.55 (Figure 6) correspondent to the hydrogen atoms of methyl ester indicated that pristimerin remained unreacted under the adopted reaction conditions. For this reason, a second attempt was performed to obtain the compound **R4** using the transesterification method described by Chavan *et al.*, (2004), in which iodine is used as catalyst. The reaction was carefully monitored by TLC, but the desired product was not obtained.

Given the inability to directly obtain compound **R4** through transesterification, an alternative approach was considered, involving the hydrolysis of pristimerin to yield celastrol, followed by esterification. It's worth noting that celastrol was not isolated from *S. crassifolia*. There are several reports of modifications in the carbon C-29 of celastrol (Shan et al., 2017; Xu et al., 2019). The first attempt to obtain celastrol from pristimerin followed the methodology described by Camelio, Johnson and Siegel, (2015), employing KOH followed by phosphate buffer (pH 2) to acidify the

reaction medium. The reaction was monitored by TLC and formation of products was not observed after 4 hours.

Alternative attempts to hydrolyze pristimerin using biocatalysts were also performed. Four different immobilized lipases, including immobilized lipase B from *Candida antarctica*, as well as the protease alpha-chymotrypsin, were employed in varied conditions, such as different solvents, temperatures, and reaction times. However, all attempts to convert pristimerin in celastrol were found to be unsuccessful (Dos Santos et al., 2024).

Steric effects might account for the stability of pristimerin toward transesterification and ordinary alkaline hydrolysis herein described. In fact, the rate of ester hydrolysis in six-membered alicyclic systems has been reported to be lower when the carbonyl moiety occupies an axial position in comparison to the equatorial position possibly due to the great steric hindrance imposed by the tetrahedral intermediate-like transition state associated with the outcome of the reaction (Burden et al., 1980; Bekkum et al., 2010). The reaction rate is lower when an axial conformation is favored, for instance, by the presence of an additional substituent on the carbon bearing the carbonyl moiety, and even lower in rigid systems with “locked” conformation, such as polycyclic, decalin-like scaffolds (Spencer et al., 1968; Burden et al., 1980). A strikingly example is the methyl ester of podocarpic acid, which axial carbomethoxy group has found to be stable under base-catalyzed and other hydrolytic conditions (Wenkert and Jackson, 1958).

Although the hydrolysis of sterically hindered esters might be achieved under harsh conditions methods suitable for hydrolysis of pristimerin are limited due to the presence of labile functional groups (Spencer et al., 1968; Wenkert and Jackson, 1958; Bekkum et al., 2010; Krief and Kremer, 2010; Camelio, Johnson and Siegel, 2015;).

Hydrolysis catalyzed by lipases known to present broad scope, such as CAL-B (Bornscheuer and Kazlauskas, 2006), was also attempt. The use of enzymes usually allows for catalysis under mild conditions and selective reactions. Unfortunately, pristimerin was found to be a particularly challenging substrate for the selected enzymes probably due to bulkiness and the fact that the carbomethoxy group is attached to a quaternary center.

Despite the rapid evolution of diagnostic methods and therapies, metastasis and resistance to administered drugs represent obstacles to the effectiveness and success of treatment. In addition to the challenge of early diagnosis and appropriate therapeutic strategies to increase the chances of the patient responding positively to treatment and increase their life expectancy, there is the issue of side effects of chemotherapy (Kubczak et al., 2021).

Due to their great chemical diversity, natural products continue to be extensively investigated as potential anticancer compounds. The efforts of researchers in phytochemistry, pharmacology and other areas of science have provided substantial advances, providing compounds for clinical use and

opening up new therapeutic opportunities. However, many challenges in oncology still remain ahead (Huang et al., 2021). In this scenario, the realization of new attempts of synthesis of pristimerin trimer using more advanced techniques and equipment, aiming its interaction with the DNA of cancer cells is considered promising.

CONCLUSION

The synthetic route used to obtain the pristimerin trimer led to the obtaining of three intermediates. The first step of the route, related to obtaining the product **R1** via an S_N2 reaction, was performed with a yield of 53%. The product with the amine group protected with Boc, compound **R2**, was obtained with 87% yield, and the trialkyne **R3** was obtained with 43% yield. Various methodologies were employed in an attempt to transesterify pristimerin with **R1** to obtain the desired product **R4**. These approaches included both-chemical and enzymatic hydrolysis of pristimerin, followed by esterification as an alternative route. However, none of these methods proved successful, underscoring the remarkable resistance of the C-29 carbon in pristimerin to hydrolysis and transesterification reactions, likely due to steric hindrance. Obtaining the pristimerin trimer for its interaction with the DNA of cancer cells is important and it is suggested that other methods and conditions of reaction continue to be investigated.

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