Ring A structural modified derivatives of withaferin A and the evaluation of their cytotoxic potential
Chapter 4

Ring A structural modified derivatives of withaferin A and the evaluation of their cytotoxic potential

1.0. Introduction:

The Solanaceae family consisting of 84 genera, about 3,000 species is found worldwide and the members of this family are generally annual shrubs. The genera Withania and Physalis is known for its use in indigenous medicines of South East Asia, e.g. in the Unani and Ayurvedic systems. There are about twenty three known Withania species, widely distributed in the drier parts of tropical and subtropical zones, ranging from the Canary Islands, the Mediterranean region and Northern Africa to Southwest Asia.\(^1\)\(^-\)\(^4\) However, only W. somnifera and W. Coagulans are economically and medicinally significant, being used and cultivated in several regions.\(^5\)\(^-\)\(^7\) W. somnifera, commonly known as Ashwagandha, is an important medicinal plant that has been used in Ayurvedic and indigenous medicine for over 3,000 years.\(^8\) In view of its varied therapeutic potential, it has also been the subject of considerable scientific attention. Ashwagandha roots are a constituent of over 200 formulations in Ayurvedha, Siddha and Unani medicines which are used in the treatment of various physiological disorders.\(^9\),\(^10\) Withania appears in WHO monographs on Selected Medicinal Plants and an American Herbal Pharmacopoeia monograph is also forthcoming.\(^11\)

---

In Ayurvedha, *W. somnifera* is widely claimed to have potent aphrodisiac, sedative, rejuvenative and life prolonging properties. It is also used as a general energy enhancing tonic known as Medharasayana, it means ‘that which promotes learning and a good memory’ and also useful in related geriatric problems.\(^{12,13}\) The plant was traditionally used to promote youthful vigor, endurance, strength and health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells. The similarity between these restorative properties and those of ginseng roots has led to Ashwagandha roots being called Indian ginseng.\(^10\) It also helps counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature ageing, emaciation, debility and muscle tension. The leaves of the plant are bitter in taste and used as an antihelmantc. The infusion is given in fever. Bruised leaves and fruits are locally applied to tumors and tubercular glands, carbuncles and ulcers.\(^{12,14}\) The roots are used as a nutrient and health restorative in pregnant women and old people. The decoction of the root boiled with milk and ghee is recommended for curing sterility in women. The roots are also used in constipation, senile debility, rheumatism, general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea.\(^{10,15}\) Today *W. somnifera* is widely cultivated in the drier parts of India (more than 4,000 ha) i.e. Manasa, Neemuch and Jawad tehsils of the Mandsaur District of Madhya Pradesh, Punjab, Sind and Rajasthan and Jammu and Kashmir.\(^6,8\) The fruits of the plant have a milk-coagulating property attributed to the pulp and husk of the berry, which has been used in the preparation of vegetable rennet ferment for cheese.\(^16\) In 1884 Sheridan Lea found upon examination that the coagulating substance is a ferment closely resembling animal rennet.\(^17\) The fruits are reported to be sedative, emetic and stomachic, blood-purifier and febrifuge, as an alterative, diuretic and bitter tonic in dyspepsia as well as a growth promoter in infants.\(^15\)

---

The leaves are used as a vegetable and as fodder for livestock.\textsuperscript{12, 18} The crude preparation of the plant has been found to be active against a number of pathogenic bacteria.\textsuperscript{19} 

\textit{W. somnifera} (L.) Dunal (Synon. \textit{Physalis somnifera} L.; \textit{Physalis flexuosa} L.) is an erect, grayish, stellate-tomentose undershrub (30-75 cm high) with long tuberous roots. Leaves are alternate or sub-opposite, broadly ovate to oblong, petiolate, sub-acute, entire, with lamina 5-10 x 2.5-7 cm (Fig.1). Flowers are small, greenish, axillary, solitary or in few-flowered cymes and bisexual. The calyx is gamosepalous with five 3-5 mm lobes, accrescent and inflated in a fruit. The corolla is campanulate, greenish-yellow with five 5-8 mm lobes. There are five included stamens. The ovary is ovoid/globose, glabrous and many ovuled. The style is filiform and stigma is 2-lobed. Fruit is a globose berry, orange-red when ripe and enclosed in the enlarged calyx. Seeds are many, discoid, yellow and reniform. The chromosome number is 2\(n = 48\).\textsuperscript{1, 2, 20} 

\textbf{2.0. Chemical constituents of \textit{Withania somnifera}:}  

The chemistry of \textit{Withania} species has been extensively studied and several groups of chemical constituents such as steroidal lactones, alkaloids, flavonoids, tannins etc. have been extracted, isolated and identified.\textsuperscript{14, 21-25} At present, more than 12 alkaloids, 40 withanolides and several sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of \textit{Withania} species. The major chemical constituents of these plants, withanolides are mainly localized

\begin{itemize}
\item \textit{Khan, M. T. J.; Ashraf, M.; Tehniyat, S.; Bukhtair, M. K.; Ashraf, S.; Ahmad W. Fitoferapia. 1993, 64, 367-370.}
\item \textit{Mozaffarian, V. Trees and shrubs of Iran; Farhange Moaser: Tehran, Iran. 2003, 874-877.}
\item \textit{Atta-ur-Rahman.; Jamal, A. S.; Choudary, M. I.; Asif, I. Phytochemistry. 1991, 30, 3824-3825.}
\item \textit{Rastogi, R. P.; Mehrotra, B. N. \textit{Compendium of Indian Medicinal Plants}; Central Drug Research Institute: New Delhi, India. 1998.}
\item \textit{Bandyopadhyay, M.; Jha, S.; Tepfer, D. \textit{Plant cell Rep.} 2007, 26, 599-609.}
\end{itemize}
in leaves and their concentrations usually ranges from 0.001 to 0.5% dry weight (DW).\textsuperscript{14, 26, 27} The withanolides are a group of naturally occurring C-28 steroidal lactones built on an intact or rearranged ergostane framework in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic structure (Fig. 2) is designated as the withanolide skeleton.\textsuperscript{28-32}

![Fig.2. Basic structure of withanolides.](image)

The withanolide skeleton may be defined as a 22-hydroxyergostan-26-oicacid-26, 22-lactone. There are many novel structural variants of withanolides with modifications either of the carbocyclic skeleton or the side chain and these have often been described as modified withanolides or ergostane type steroids related to withanolides. These compounds are generally polyoxygenated and it is believed that plants elaborating them possess an enzyme system capable of oxidizing all carbon atoms in a steroid nucleus. The characteristic feature of withanolides and ergostane-type steroids is one C-8 or C-9-side chain with a lactone or lactol ring but the lactone ring may be either six membered or five membered and may be fused with the carbocyclic part of the molecule through a carbon-carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to

\textsuperscript{26} Atal, C. K.; Gupta, O. P.; Ranghunathan, K.; Dhar, K. L. Central Council for Research in Indian Medicine and Homeopathy, New Delhi, India. \textit{1975}.


bond scission, formation of new bonds, aromatization of rings and many other kinds of rearrangements resulting in compounds with novel structures.\textsuperscript{28, 30, 33} Withaferin A (Fig.3) was the first member of this group of compounds to be isolated from the well known South-Asian medicinal plant \textit{W. somnifera}. The structural novelty and interesting biological activities elicited by this compound led to a thorough chemical investigation of the plant and numerous compounds with similar structural features were isolated.\textsuperscript{28, 30, 34}

![Withaferin A (1)](image)

\textbf{Fig.3.} Withaferin A (1).

Lavie’s group\textsuperscript{35} elucidated the structure of withaferin A in leaves of this plant, which is mainly valued for its anti-cancerous properties. The yields of withaferin A from intact plants of \textit{Withania} spp. (Israel chemotype) are 0.2-0.3\% of DW of leaves.\textsuperscript{36} Gupta \textit{et al.}\textsuperscript{37} have performed a quantitative analysis of Indian chemotypes of \textit{W. somnifera} by TLC densitometry and observed that withaferin A is totally absent in roots, stems, seeds and persistent calyx of fruits of intact plants but present in leaves (1.6\%). Today over 130 withanolides from Solanaceae genera are known, mostly occurring in free form, but in a few cases also as glycosides,\textsuperscript{28} some of which are shown in Fig. 4.

\begin{itemize}
  \item \textsuperscript{34} Leet, J. E.; Hussain, S. F.; Minard, R. D.; Shamma, M. \textit{Heterocycles}. \textbf{1982}, \textit{19}, 2355-2360.
\end{itemize}
Withanolide D (2); $R_1 = H, R_2 = OH, R_3 = H$; 27-deoxywithaferin A (3); $R_1 = H, R_2 = H, R_3 = H$; 14α-OH, $R_1 = H, R_2 = H$; 17α-OH, $R_1 = H, R_2 = H, R_3 = H$; 27-Hydroxywithanolide D (4); $R_1 = OH, R_2 = OH, R_3 = H$; 14α-OH, $R_1 = H, R_2 = H, R_3 = H$; 17α-OH, $R_1 = OH, R_2 = H, R_3 = H$; Dihydrodeoxywithaferin A (5); 2,3-diH, $R_1 = H, R_2 = H$; Dihydrowithaferin A 2,3-diH (6); $R_1 = H, R_2 = OH, R_3 = H$; 17-hydroxywithaferin A (7), $R_1 = R_3 = OH, R_2 = H$.

Withanolide E (8); 5β, 6β-eoxy; Withanolide F (9) $\Delta^5$; Withanolide S (10), 5α-OH, 6β-OH.

Withanolide G (11); 14α-OH, 14 = OH, $R_1 = OH, R_2 = H$; Withanolide H (12); 14α-OH, $R_1 = R_2 = OH$; Withanolide J (13); 7α-OH $R_1 = H, R_2 = OH$; 17α-OH $R_1 = H, R_2 = OH$; 17α-OH, $R_1 = OH R_2 = H$; $\Delta 16$-withanolides (14); $R_1 = OH, R_2 = H$.

**Fig.4.** Different structures of withanolides.
Chapter 4

2.1. Other chemical constituents of *Withania somnifera*:

Examination of *W. somnifera* roots has resulted in the isolation of a new dimeric thiowithanolide named as Ashwagandhanolide (18) (Fig.5).\(^{38}\)

![Fig.5. Ashwagandhanolide (18) from *W. Somnifera.*](image)

A bioassay guided purification of the methanolic extract of *W. somnifera* fruits yielded withanamides 19A-H (Fig.6). The structures of these compounds were determined by using serotonin, glucose and long-chain hydroxyl fatty acid moieties.\(^{39}\) In their quantitative analysis of Indian chemotypes of *W. somnifera* by TLC densitometry, Gupta *et al.*\(^{37}\) detected alkaloids in all the above mentioned plant parts with the highest content found in leaves. This is in contrast to the general belief that tropane alkaloids are restricted to the roots of *Withania* spp. Extraction with 45% alcohol yields the highest percentage of alkaloids. The isolation of nicotine, somniferine, somniferinine, withanine, withanamine, pseudowithanine, tropine, pseudotropine, 3α-tigloyloxytropane, choline, cuscohygrine, *dl*-isopelletierine and new alkaloids anaferine and anhygrine has been described.\(^{14, 40}\) The reported total alkaloid content in the roots of Indian *W. somnifera* varies between 0.13 and 0.31%, though much higher yields (upto 4.3%) have been recorded in plants of other

---

regions/countries. In addition to the alkaloids, the roots are reported to contain starch, reducing sugars, hentriacontane, glycosides, dulcitol, withanicil, an acid and a neutral compound.\textsuperscript{40} The leaves are reported to contain five unidentified alkaloids (yield 0.09\%), chlorogenic acid, calystegines (nitrogen-containing polyhydroxylated heterocyclic compounds) withanone, condensed tannins and flavonoids. The berries have amino acids. Four types of peroxidases have been purified and characterized from \textit{W. somnifera} roots.\textsuperscript{14, 41}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{withanamides.png}
\caption{Different withanamides (A-H) isolated from \textit{W. somnifera} fruits.}
\end{figure}

3.0. Pharmacological activities of withanolides and withaferin A:

The pharmacological activity of *W. somnifera* extracts has been summarized recently by Gupta and Rana. Historically, *W. somnifera* has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent and astringent and more recently as an antibacterial, antihyperglycemic and antitumoral as well as in the treatment of ulcers and senile dementia. The active principles of *W. somnifera* have been tested for antioxidant activity by observing the levels of the major free-radical scavenging enzymes, superoxide dismutase, catalase and glutathione peroxidase in the rat brain frontal cortex and striatum. The increase in these enzymes after treatment with withanolides represent enhanced antioxidant activity and a corresponding protective effect on neuronal tissue, suggesting that the antioxidant effect of *W. somnifera* in the brain may be responsible for its diverse pharmacological properties. Similarly, oral administration of *W. somnifera* extracts prevented an increase in lipid peroxidation in mice and rabbits. Anxiolytic and antidepressant actions of the bioactive withanolides have been assessed in rats. Withanolides reduced rat brain levels of tribulin (an endocoid marker of anxiety) when the levels of this compound were increased by administration of pentylenetetrazole, an anxiogenic agent. The antidepressant effect of withanolides is comparable with that induced by imipramine in the forced swim induced “behavioural despair” and “learned helplessness” test. In a rat model, the withanolides were able to decrease the number and severity of chronic stress induced ulcers, reverse chronic stress induced inhibition of male sexual behavior, chronic stress induced immunosuppression and also increased peritoneal macrophage activity. It has also been demonstrated that methanolic extracts of *Withania* reduced ulcer index, volume of gastric secretion, free acidity and total acidity. The effects of sitoindosides VII-X and withaferins isolated from aqueous methanol extracts of *W. somnifera* roots were studied on brain cholinergic, glutamatergic and GABAergic receptors in rats. The data suggest the bioactive compounds preferentially influence events

in the cortical and basal forebrain cholinergic signal transduction cascade. The cognition enhancing and memory improving effects of *W. somnifera* extracts can be partly explained by the drug induced enhancement of cortical muscarinic acetylcholine receptor capacity.\(^{47}\) In general, Ashawagandha has been used traditionally and generally as a tonic and nootropic agent. It has also been associated with improvements in scopolamine-induced memory deficits in mice.\(^{48}\) The methanolic extracts of the plant comprising withanolides such as withanolide A, withanoside IV and withanoside VI have been reported to induce neurite extension,\(^{49}\) inducing neurite outgrowth in human neuroblastome SHSY5Y.\(^{50}\) Dendritic atrophy was completely prevented by treatment with withanolides like withanolide A, withanoside IV and withanoside VI and more particularly by withanoside IV and VI.\(^{51}\) Neuroleptic induced catalepsy has been used as an animal model for screening drugs for Parkinson’s disease. Hopes of the treatment for this disease has been enhanced by the inhibitory effects of *W. somnifera* extracts on haloperidol or reserpine induced catalepsy in mice.\(^{52}\) The antiparkinsonian effect of *W. somnifera* extracts has also been attributed to potent antioxidant, antiperoxidative and free radical quenching properties.\(^{53}\) The extracts of *W. somnifera* have shown antiinflamatory effects in a variety of rheumatological conditions reducing, for example, Freund’s complete adjuvant induced inflammation in rats and decreasing to undetectable levels the a2-glycoprotein found only in inflamed rat serum.\(^{54}\) In another study, *W. somnifera* caused suppression of alpha-2-macroglobulin, an indicator for anti-inflammatory drugs in rat serum inflamed by injection of carrageenan suspension.\(^{55}\) The extracts also caused a significant reduction in both paw swelling and bony degenerative changes in Freund’s adjuvant induced arthritis as observed

---


by radiological examination. \(^{56}\) Rats injected with formaline in the hind leg footpad showed decreased absorption of 14C-glucose in rat jejunum; the glucose absorption being maintained at the normal level by *Withania* extracts, which produced an anti-inflammatory effect. \(^{57}\) The traditional antihyperglycemic and antidyslipidemic activities of *W. coagulans*, popularly known as Indian cheese marker have been recently confirmed. \(^{58}\) Withanolides isolated from *W. coagulans* fruits show significant inhibition of the postprandial rise in hyperglycemia post-sucrose load in normoglycemic rats as well as streptozocin induced diabetic rats. *W. somnifera* extracts have a chemopreventive effect on the skin cancer in mice induced by 7,12- dimethylbenz[a]anthracene. This activity is thought to be partly due to the antioxidant/free radical scavenging activity of the extract. \(^{59}\)

The bioactive properties of withaferin A such as cytoskeletal architecture alteration by covalently binding annexin II, \(^{60}\) antitumor capacity by inhibition of proteasomal chymotrypsin like activity \(^{61}\) and apoptosis induction through the inhibition of protein kinase C have also been reported. \(^{62}\) In relation to the apoptosis inducing mechanism of withaferin A, Oh *et al.* \(^{63}\) have demonstrated that it is associated with the activation of caspase-3 and the translocation of cytochrome *c* from the mitochondria to the cytosol as well as the cleavage of PLC-γ1 (a substrate protein of caspases), whereas ectopic expression of Bcl-2 oncoprotein significantly attenuates withaferin A induced apoptosis.

---

4.0. Structural features of withaferin A:

Withaferin A (4β,27-dihydroxy-1-oxo-5β,6β-epoxywitha-2-24-dienolide 1) is a polyfunctional steroidal lactone with 28 carbon atoms based on ergostane framework (Fig.7). Chemically, there are 4 major functionalities in withaferin A: (i) α, β-unsaturated ketone group in ring A, (ii) a secondary hydroxyl group at C-4, (iii) a labile epoxide ring between C-5, C-6, (iv) a primary hydroxyl group at C-27 and (v) a 6-membered lactone ring (E) with α, β-unsaturated carbonyl group.

![Diagram of Withaferin A](image)

**Fig.7.** Functionalities in withaferin A (1).

5.0. Literature reports for the structural modifications of withaferin A:

There are only a few reports available on the structure modification work on withaferin A owing to the presence of multiple reactive sites. Much emphasis has been given to the hydroxyl groups at C-27 and 5β,6β-epoxide group towards antiproliferative activity of withaferin A (1).

For example, Yasuno Yokota et al.\textsuperscript{64} developed probes for studying angiogenesis and to identify the biological target for Withaferin A (1) using hydroxyl group at C-27. They generated biotinylated affinity analogue WFALC2B (21) as probes and showed its inhibiting character in the angiogenic sprouting in vitro conditions and suppressing the activity of withaferin A (1). From the study the researchers conclude that 21 acted as probe for identifying the biological target of 1. The reaction procedure involves coupling of 1 with Fmoc-Gly-OH to generate the intermediate Fmoc-Gly-WFA (20), purification of the same and subsequent deprotection of Fmoc group followed by reaction with NHS-LC-LC-biotin to generate 21(Scheme-1).

---

Scheme-1. Reagents and conditions: (a) i. Fmoc-Gly-OH, oxalyl chloride/DMF, CH₂Cl₂, rt., 3h; ii. DMAP, CH₂Cl₂, rt., 3h; (b) i. 20% piperidine/DMF (15 min); ii. NHS-LC-LC-biotin, DMSO, rt., 30 min. Laxminarain Misra et al.⁶⁵ reported the reaction of 2-mercaptoethanol (22) selectively with 5β,6β-epoxy ring of 1 (Scheme-2) towards the synthesis of six-membered oxyethylene-2-thio ring (23) and concluded that 5,6-epoxy ring is necessary for the biological activity of withaferin A.

Scheme-2. Reaction of withaferin A with 2-mercaptoethanol.

Allan et al.⁶⁶ reported the reaction of withaferin A with three biological nucleophiles like thiophenol, ethyl mercaptan and L-cysteine ethyl ester. However, the authors concluded that the role of the A-ring double bond of withaferin A in antitumour and or cytotoxic activity is unclear.

---

6.0. Objectives of the present Work:
The presence of steroidal structural framework in natural products has opened new vistas for medicinal and biological chemistry. They are part of the cell membrane and their significance can be attributed to essential biological functions including adrenal and sex hormones, bile acids, precursors of certain vitamins as well as their roles in steroid based chemotherapeutics such as inflammations etc. Steroidal framework provides a template for structural modifications as these are generally functional group rich chemical entities. Moreover, these biologically significant motifs are reported to be non-toxic, less susceptible to multidrug resistance (MDR) and highly bio-available owing to their capability to penetrate the biological membranes.67 This is also true to the skeleton with an α, β-unsaturated function whereby the assimilation of heteroatom (S, N & O) has been reported to augment or modulate the biological activities of newly generated chemical entities.68 Literature is full of examples underscoring natural products with α, β-unsaturated carbonyl moiety exhibiting cancer chemo-preventive and chemo-protective activities (Fig.8).68 The hallmarks of the biological profile of α, β-unsaturated carbonyl compounds is their ability to act as Michael acceptors.69 Although traditionally shunned in modern drug discovery,70 trapping of nucleophiles like thiols by covalent coupling represents an important mechanism of biological activity. This has lead to the discovery of many biologically relevant pathways to understand the mechanism of action of the particular drug candidate. Research on Michael acceptors, long confined to the realm of toxicology71 was rekindled by the development of the antioxidant inflammation modulator homoterpenoid bardoxolone methyl (RTA402)72 that was given the orphan drug status by the FDA for the treatment of pancreatic cancer. Currently in drug development, irreversible binding in the active sites has proven to be one answer to drug resistance in cancer treatment.

Fig.8. Structures of α,β-unsaturated carbonyl compounds that show biological activity.

Thus, in order to include the α, β-unsaturated carbonyl group in drug development strategies, the potentially adverse effects must be circumvented. This can be achieved by fine tuning the reactivity of the α, β-unsaturated carbonyl unit leading to a high degree of specificity. To accomplish this goal, it is essential to understand what reactivity is in place within a molecule. Different approaches comparing similar natural products and synthetic molecules have been followed in order to predict the reactivity of α, β-unsaturated carbonyl compounds. The α, β-unsaturated carbonyl moiety permits the direct adjustment of electrophilicity and reduction potential through modification of the peripheral substituent. Substitution of the α,β-unsaturated carbonyl system at the β-position has yet to be extensively addressed and may be a practical approach to modify biological activity. It may even be possible to find new activities of the modified compounds.
In this regard, withaferin A (1), a steroidal lactone from Withania somnifera (Aswagandha) has attracted the attention of chemists as well as biologists due to its interesting structure and reported anti-cancer, adaptogenic, anti-stress, anti-convulsant, immunomodulatory and neurological effects. It is known to be a natural proteosome inhibitor, apoptotic agent inducing apoptosis by inhibiting topoisomerase I-DNA complex and act as mitotic poison along with its antiangiogenic potential. Even though, the actual mode of action of withaferin A remains ambiguous and may be specific to cell-types. Recently, it has been reported to induce actin microfilament aggregation mediated by annexin-II.

There are only few reports available on the modification work of withaferin A owing to presence of multiple reactive sites. Much emphasis has been given to the hydroxyl groups at C-27 and 5β, 6β-epoxide group towards antiproliferative activity of withaferin. Only report available in the literature showed that ring A analogue of withaferin A exhibited sarcoma 180 activity in mice, however, the conflicting results have made it difficult to ascertain the importance of ring A. Thus, a detailed investigation of the importance of α, β-unsaturated group in ring A is much desirable. Recently, it has been reported that 2, 3-dihydro withaferin A-3β-O-sulphate acts as a potent prodrug of withaferin A (1). In the

---

light of promising therapeutical prospective of withaferin A (1) as reported by our parent institution as anticancer molecule, we became interested in the study of modifications of ring A. We envisaged that substitution on the α,β-unsaturated carbonyl system of withaferin A at the β-position may be a practical approach to find new activities of the modified compounds as this will obviate irreversible covalent binding in active sites by biological nucleophiles, a reason behind drug resistance in cancer treatment. Thus, in the present chapter, we describe a regio- and stereoselective addition of ring A in the presence of multiple electrophilic sites, evaluation of cytotoxicity of the so generated library and further modification of the most active derivatives for the pursuit of anticancer lead molecules.

7.0. Results and discussions:

7.1. Structural modification of withaferin A (1) at ring A:

In order to comprehend the role of double bond in the ring A of withaferin A (1) for anticancer activity, we envisaged to study the effect of Michael addition to α, β-unsaturated carbonyl group of ring A. Accordingly, a solution of withaferin A in methanol was treated with a basic solution of nucleophile TMSN₃ (36) at room temperature (Scheme-3). For the optimization of the reaction conditions, the addition reaction was carried out at varying pH using different organic and inorganic bases (Table-1). It was observed that a pH of 8-8.5 is the optimal for the reaction operation. Among the various bases used, the most favourable was triethylamine in terms of yield, reaction time and minimal side product formation. After the formation of the product and processing, it was purified through silica gel (60-120 mesh size) column chromatography.

Scheme-3. Reaction of Withaferin A (1) with TMSN₃ (36).

Table-1. Standardisation of reaction conditions for Michael addition.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Base used</th>
<th>pH</th>
<th>Time (h)</th>
<th>Yield%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaOAc</td>
<td>7.5</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>DIPEA</td>
<td>7.5</td>
<td>6</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5</td>
<td>6</td>
<td>53</td>
</tr>
<tr>
<td>3</td>
<td>Et₃N</td>
<td>7.5</td>
<td>3.5</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5</td>
<td></td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.5</td>
<td></td>
<td>65°*</td>
</tr>
<tr>
<td>4</td>
<td>C₅H₅N</td>
<td>7.5</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>pyrrolidine</td>
<td>7.5</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.5</td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

*Some degradation of starting material along with the desired product was noticed.
Chapter 4

The structure of the product 37 was elucidated by spectroscopic methods and a comparison was made between 1 and 37 (Table-2).

**Table-2.** Comparison of $^1$H NMR and $^{13}$C NMR of azide derivative (37) and withaferin A (1).

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Compound 1 $^1$H NMR (CDCl$_3$, 500 MHz)</th>
<th>$^{13}$C NMR</th>
<th>Compound 37 $^1$H NMR</th>
<th>$^{13}$C NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>--</td>
<td>202.48</td>
<td>--</td>
<td>208.49</td>
</tr>
<tr>
<td>2</td>
<td>6.21 (d, J = 10 Hz)</td>
<td>132.42</td>
<td>3.07 (dd, J = 7.3, 15.7 Hz, 2H)</td>
<td>38.75</td>
</tr>
<tr>
<td>3</td>
<td>6.98 (dd, J = 10.0, 6.0 Hz)</td>
<td>142.47</td>
<td>4.08 (ddd, J = 15.7, 4.1, 3.3 Hz, 1H)</td>
<td>58.87</td>
</tr>
<tr>
<td>4</td>
<td>3.76 (d, 1H, J = 6.0 Hz)</td>
<td>69.85</td>
<td>3.46 (d, J = 4.1 Hz, 1H)</td>
<td>75.15</td>
</tr>
<tr>
<td>5</td>
<td>--</td>
<td>63.99</td>
<td>--</td>
<td>65.10</td>
</tr>
<tr>
<td>6</td>
<td>3.24 (brs, 1H)</td>
<td>61.80</td>
<td>3.26 (brs, 1H)</td>
<td>59.85</td>
</tr>
<tr>
<td>21</td>
<td>1.02 (d, J = 7.0 Hz, 3H)</td>
<td>13.31</td>
<td>1.01 (d, J = 6.9, 3H)</td>
<td>13.34</td>
</tr>
<tr>
<td>22</td>
<td>4.40 (dt, J = 12.0, 3.5 Hz)</td>
<td>78.78</td>
<td>4.40 (dt, J = 12.0, 3.5 Hz)</td>
<td>78.73</td>
</tr>
<tr>
<td>23</td>
<td>2.50 (brs, 2H)</td>
<td>28.82</td>
<td>2.54 (m, 2H)</td>
<td>29.68</td>
</tr>
<tr>
<td>24</td>
<td>--</td>
<td>153.71</td>
<td>--</td>
<td>153.18</td>
</tr>
<tr>
<td>25</td>
<td>--</td>
<td>125.57</td>
<td>--</td>
<td>125.61</td>
</tr>
<tr>
<td>26</td>
<td>--</td>
<td>167.23</td>
<td>--</td>
<td>167.68</td>
</tr>
<tr>
<td>27</td>
<td>4.35 (brs, 2H)</td>
<td>56.97</td>
<td>4.30 (brs, 2H)</td>
<td>57.33</td>
</tr>
<tr>
<td>28</td>
<td>2.02 (s, 3H)</td>
<td>20.08</td>
<td>2.05 (s, 3H)</td>
<td>20.04</td>
</tr>
</tbody>
</table>

$^1$H NMR spectrum of the product 37 showed set of signals similar to those in the spectrum of the 1 except the two additional signals as dd at $\delta$ 3.07 for H-2 and ddd at $\delta$ 4.08 for H-3. The disappearance of characteristic signals at $\delta$ 6.17 (d, J = 10.0 Hz), and $\delta$ 6.96 (dd, J = 6.0, 10.0 Hz) for H-2 and H-3 respectively corresponding to $\alpha$, $\beta$-unsaturated carbonyl group in the $^1$H NMR of 1 was a clear indication of the formation of Michael adduct 37. The signal for H-4 $\delta$ 3.46 (d, J = 4.1 Hz, 1H) was shifted slightly upfield in the 1H spectrum of 37 as compared to 1 as expected. These assignments which evidently differed from those of 37 were confirmed by using various techniques of 2D NMR. In the $^1$H-$^1$H COSY plot of 37, H-3 at $\delta$ 4.08 showed correlations with H-4 and H-2a/b at $\delta$ 3.46 and 3.07 respectively. Further strong correlations were found between H-2 and H-4. In the $^{13}$C NMR spectrum, two additional signals appeared at 38.75 and 58.87 for C-2 (CH$_2$) and C-3 (CH) respectively along with the downfield shift of C-4 to $\delta$ 75.15. These interpretations were supported by the HMBC and HSQC spectrum of 37 also. In HMBC spectra of 37, the
proton signal at δ 4.08 showed a cross peak with the carbon signal at δ 65.10 (C-5), 208.49 (C-1) and the proton signal at δ 3.07 showed a cross peak with the carbon signal at δ 75.14 (C-4) also the proton signal at δ 3.64 (H-4) showed a strong correlation with δ 65.10 (C-5), 59.85 (C-6), 38.75 (C-2). The HMBC data indicated the position of azide group, hydroxyl group and an epoxy group (Fig. 9). The HSQC spectrum of 37 showed that the carbon signal resonating at δ 58.87 had a correlation with H-3 at δ 4.08 while in HMBC, this signal showed correlation with H-2 and H-4 supporting that azide group is present at C-3 without the typical unsaturation at C-2,3. As reported by Choudhary et al.\textsuperscript{81} the stereochemistry at C-3 was assigned as β since the ddd at 4.08 Hz showed $J = 3.3, 4.1, 15.7$ Hz which represents the characteristic $J$ values for β-orientation. It is noteworthy that while addition to the α, β-unsaturated carbonyl group of 1, neither the reactive epoxide group reacted nor the lactone was disturbed. This became apparent by the fact that the signals for epoxide group at 5,6 position at δ 3.26 (bs for H-6 in $^1$H NMR of 37) and δ-lactone moiety at δ 78 ppm in $^{13}$C NMR were intact.

Chapter 4

7.2. **Reaction of withaferin A (1) with thiol nucleophiles:** After optimising the reaction conditions for Michael addition of withaferin A, next thiol nucleophiles were tested for their reactivity and selectivity. First thiophenol was allowed to react with withaferin A under the standardised reaction procedure. It was found that complete conversion of starting material occurred within 3 h. The product was characterised through spectroscopic analysis and was assigned the structure 38. Other thiol nucleophiles like chlorothiophenol, p-methoxy thiophenol and furan ethanethiol reacted smoothly to yield the corresponding Michael adducts (39-42) in good to excellent yields (Scheme-4).

![Scheme-4. Reaction of thiol nucleophiles with 1.](image)

7.3. **Reaction of withaferin A (1) with amino acids:**
Amino acids are crucial for life and are involved in many metabolic functions. In general these act as building blocks of proteins. Chemically these are bi-functional organic molecules with one amino group and one carboxylic group. The amino group is the nucleophilic centre and carboxylic group is the electrophilic centre. Thus, during the process of using these as nucleophiles, the carboxylic functionality needs to be protected. Hence, in all the cases methyl esters of amino acids were used. Thus, methyl ester of phenylalanine was allowed to react for 4 h with 1 under the standardised reaction conditions to get the corresponding Michael adduct 43 (Scheme-5).
Scheme-5. Reaction of methyl esters of amino acids with 1.

The product was characterized through spectroscopic analysis. Similarly other amino acids like valine methyl ester and cysteine were subjected to reaction with withaferin A to prepare the amino acid derivatives (44, 45). Since the nucleophilicity of thio centre in cysteine is more compared to the amino group, thus, the thio addition product 45 was obtained which was fully characterised by $^1$H NMR and $^{13}$C NMR (Scheme-6).

Scheme-6. Reaction of cysteine methyl ester with 1.

7.4. Huisgen’s 3+2 cycloaddition of Michael adduct 37:

The Huisgen 1,3-dipolar cycloaddition reaction of organic azides and alkynes has gained considerable attention in recent years due to the introduction of Cu(I) catalysis in 2002 by Tornøe and Meldal, leading to a major improvement in both rate and regioselectivity of the reaction, as realized independently by the Meldal and the Sharpless laboratories. The great success of the Cu(I) catalyzed reaction is rooted in the fact that it is a virtually quantitative, very robust, insensitive, general and orthogonal ligation reaction, suitable for even biomolecular ligation and in vivo tagging or as a polymerization reaction for synthesis of long linear polymers. The “click chemistry” term, first proposed by Sharpless et al. has been coined for the reactions that, “are modular, wide in scope, high
yielding, create only inoffensive by-products (that can be removed without chromatography), are stereospecific, simple to perform and that require benign or easily removable solvent.”

With high regioselectivity, 100% atom economy and convenient product isolation, the click version of Huisgen [3+2] cycloaddition has been increasingly used in various applications ranging from chemical and combinatorial synthesis, bioconjugation and biology to material science, especially polymer and dendrimer synthesis. It serves as a tool for the synthesis of various small molecule inhibitors exhibiting a broad spectrum of biological activity including anti-HIV, anti-allergic, anti-bacterial, herbicidal, fungicidal and anti-haemagglutination activity. Furthermore, it is worth noting that triazoles have become important pharmacophores in contemporary drug discovery because they are stable, safe and could mimic peptide bonds from a steric and electronic point of view without the same sensitivity towards hydrolytic cleavage.

84. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, B. K. Angew. Chem. Int. Ed. 2002, 41, 2596-2599.
Chapter 4

The reaction involves a stepwise Cu (I)-catalyzed dipolar cycloaddition of an organic azide (46) and a terminal acetylene (47) to form exclusively 1,4-disubstituted-1,2,3-triazoles (48) (Scheme-7).

Scheme-7. Cu (I)-catalysed 1,3-dipolar cycloaddition of azides and terminal alkynes.

Owing to the biological importance of triazoles and simplicity of the reaction conditions, we next synthesised the triazole derivatives of 3-β-azido analogue (37) of 1. Also, these derivatives are expected to have more water solubility than the parent compound.

In our initial studies, methyl propiolate (49) was used as the alkyne partner in the cycloaddition reaction. Thus, to a solution of 37 in acetonitrile was added methyl propiolate (49) in equimolar ratio followed by addition of copper iodide (CuI) as [Cu(I)] source (Scheme-8).

Scheme-8. Cu(I) catalysed 1,3-dipolar cycloaddition reaction of azide derivative 37 and methyl propiolate.

The reaction mixture was allowed to stir at room temperature and monitored through TLC. The consumption of starting material indicated the completion of the reaction after 6 h. After usual workup the product 50 was obtained in 99% yield. Accordingly, other triazole derivatives were synthesised applying the same reaction procedure using other alkynes (Scheme-9). The carbohydrate based alkyne partners were found to react smoothly under these reaction conditions to afford the triazole glycoconjugates 53, 54.
In order to study the effect of unprotected hydroxyls on the cytotoxicity, the triazole derivative 54 was deacetylated using NaOMe in methanol to achieve the synthesis of triazole glycoside 55.

7.5. In vitro screening of structurally modified withaferin A analogues for cytotoxicity:

7.5.1. Sulphorhodamine B assay for % growth inhibition:

The sulphorhodamine B (SRB) assay was used to screen the semi-synthetic library of withaferin A for cytotoxicity. The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue-culture plates by trichloroacetic acid (TCA). SRB is a bright pink aminoxanthene dye with two sulphonic groups that bind to basic amino-acid residues under mild acidic conditions and dissociates under basic conditions. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell mass.

In order to determine the effect of the synthesised compounds on cell number over time, SRB assays were performed as described. Various human cancer cell lines which included lung, ovary, colon and prostate were seeded in flat-bottomed 96-well plates. The cells were allowed to adhere overnight and then media containing samples at different concentrations
were added. The plates were assayed for 48 h. The cells were fixed by adding 50 mL per well of ice-cold 50\% TCA to each well for 60 min. The plates were washed five times in running tap water and stained with 100 mL per well SRB reagent (0.4\% w/v SRB in 1\% acetic acid for 30 min). The plates were then washed five times in 1\% acetic acid and allowed to dry overnight. SRB was solubilised with 100 mL per well 10 mM tris-base, shaken for 5 min. and the OD was measured at 570 nm with reference wavelength of 620 nm. The results are given in Table-3.

---

Table 3. *In vitro* determination of cytotoxicity of structurally modified derivatives of withaferin A against panel of human cancer cell lines.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Compound</th>
<th>Conc.(µM)</th>
<th>Lung</th>
<th>Ovary</th>
<th>Colon</th>
<th>Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>100</td>
<td>95</td>
<td>90</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>100</td>
<td>98</td>
<td>98</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>100</td>
<td>95</td>
<td>96</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>100</td>
<td>95</td>
<td>96</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>10</td>
<td>97</td>
<td>98</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>41</td>
<td>100</td>
<td>57</td>
<td>55</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>100</td>
<td>54</td>
<td>76</td>
<td>93</td>
<td>57</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
<td>100</td>
<td>81</td>
<td>69</td>
<td>99</td>
<td>88</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
<td>100</td>
<td>86</td>
<td>79</td>
<td>99</td>
<td>94</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>100</td>
<td>94</td>
<td>90</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>11</td>
<td>46</td>
<td>100</td>
<td>96</td>
<td>88</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>10</td>
<td>62</td>
<td>83</td>
<td>97</td>
<td>80</td>
</tr>
<tr>
<td>13</td>
<td>48</td>
<td>100</td>
<td>90</td>
<td>86</td>
<td>88</td>
<td>86</td>
</tr>
<tr>
<td>14</td>
<td>49</td>
<td>100</td>
<td>95</td>
<td>90</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>100</td>
<td>95</td>
<td>86</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>16</td>
<td>51</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td>17</td>
<td>Paclitaxel</td>
<td>60</td>
<td>92</td>
<td>86</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>18</td>
<td>Mitomycin</td>
<td>96</td>
<td>79</td>
<td>62</td>
<td>62</td>
<td>45</td>
</tr>
<tr>
<td>19</td>
<td>5-Fluorouracil</td>
<td>76</td>
<td>60</td>
<td>61</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Adriamycin</td>
<td>83</td>
<td>85</td>
<td>76</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>
The cytotoxicity data in Table-3 clearly indicated that out of 16 compounds 10 molecules are found active in primary screening. These 10 molecules i.e. 1, 37, 38, 39, 45, 50, 51, 53, 54 and 55 are the most active on all the four cancer cell lines that is lung, ovary, colon and prostate. Subsequently all the 10 molecules are further screened at lower micro molar concentrations (Table-4).

**Table-4. Cytotoxicity of selected compounds 1, 37, 38, 39, 45, 50, 51, 53, 54 and 55 on five cancer cell lines.**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Inst. Code</th>
<th>Conc. (µM)</th>
<th>Prostate (PC-3)</th>
<th>Ovary (IGR-OV-1)</th>
<th>Breast (MCF-7)</th>
<th>Cervix (HeLa)</th>
<th>Leukemia (THP-1)</th>
<th>% growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>64</td>
<td>54</td>
<td>71</td>
<td>38</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>1</td>
<td>65</td>
<td>85</td>
<td>79</td>
<td>0</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>1</td>
<td>62</td>
<td>66</td>
<td>75</td>
<td>0</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>1</td>
<td>58</td>
<td>68</td>
<td>66</td>
<td>16</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>1</td>
<td>60</td>
<td>82</td>
<td>63</td>
<td>0</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>1</td>
<td>54</td>
<td>58</td>
<td>71</td>
<td>6</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>1</td>
<td>14</td>
<td>34</td>
<td>54</td>
<td>37</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>53</td>
<td>1</td>
<td>56</td>
<td>49</td>
<td>60</td>
<td>2</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>54</td>
<td>1</td>
<td>65</td>
<td>68</td>
<td>80</td>
<td>9</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>1</td>
<td>37</td>
<td>68</td>
<td>64</td>
<td>9</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>5-Fluorouracil</td>
<td>1</td>
<td>54</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Mitomycin</td>
<td>1</td>
<td>-</td>
<td>56</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Paclitaxel</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The cytotoxicity result shown above indicated that compounds 1, 37, 38 and 54 maintained their cytotoxicity at lower micro molar concentrations on all the four cancer cell lines that is prostate, ovary, breast and leukemia where as the cervical cancer cell line was fairly resistant to the cytotoxic effect of the entire 10 compounds. The prostate and ovarian cancer cell lines became resistant to the action of the compounds 39, 45, 46, 51, 53 and 55 respectively, where as these compounds maintained their cytotoxicity on the breast and leukemia cancer cell lines. Since the compounds 1, 37, 38 and 54 are the most active on all the cancer cell lines, so these three molecules are further evaluated at still lower micro molar concentration (Table-5).
### Table-5. Cytotoxicity of compounds 1, 37, 38 and 54 on six cancer cell lines.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Lung</th>
<th>Ovary</th>
<th>Prostate</th>
<th>CNS</th>
<th>Colon</th>
<th>Leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line Type</td>
<td>A-549</td>
<td>IGR-OV-1</td>
<td>PC-3</td>
<td>IMR-32</td>
<td>CoLo-205</td>
<td>THP-1</td>
</tr>
<tr>
<td>S.No.</td>
<td>Compound</td>
<td>Conc.(µM)</td>
<td>% growth inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>---------</td>
<td>------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Paclitaxel</td>
<td>1</td>
<td>77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Mitomycin</td>
<td>1</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Adriamycin</td>
<td>1</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The calculated IC$_{50}$ values of the best four compounds on the various human cancer cell lines like lung, ovary, CNS, colon and leukemia are given in the Table-6.

### Table-6. IC$_{50}$ values 1, 37, 38 and 54 on various cell lines.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Lung</th>
<th>Ovary</th>
<th>Prostate</th>
<th>CNS</th>
<th>Colon</th>
<th>Leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Line Type</td>
<td>A-549</td>
<td>IGR-OV-1</td>
<td>PC-3</td>
<td>IMR-32</td>
<td>CoLo-205</td>
<td>THP-1</td>
</tr>
<tr>
<td>S.No.</td>
<td>Compound</td>
<td>IC$_{50}$ values(µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>-------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 7.5.2. General SAR of withaferin A derivatives:

Overall the data obtained showed that the majority of the compounds were able to induce growth inhibition in all the cell lines tested and preliminary structure-activity relationships can be inferred. The most active product was the compound 37 (IC$_{50}$ = 0.02-1.9 µM) which has an azide group at C-3 with hydroxyl group at position C-4 and epoxide group at C-5,6 intact. The presence of azide group at C-3 led to a 35-times increase in activity when compared to the parent molecule in case of IGROV-1 cell line (1 vs 37, Table-6). Among
the compounds 38-42 with different thiol groups, the 38 with thiophenol as the substituent showed the better activity (IC\textsubscript{50} = 0.13-2.2) as compared to other thiol containing compounds 39-42. Electron withdrawing groups showed a positive effect on biological activity with chloro- being more active than bromo-, while electron donating groups tend to decrease the activity. As compared to the parent molecule, compounds 43-45 bearing amino acid functionality at C-3 showed no promising cytotoxicity. Among these 45 with cysteine methyl ester as the substituent was found to be better. On considering the compounds with triazole moiety, it was observed that the activity of the compounds depended upon the side chain with 54 bearing a galactose moiety showing analogous cytotoxicity when compared to parent molecule. Increasing the hydrophilicity by deacetylation of 54 to get 55 showed deleterious effects on the growth inhibition.

8.0. Conclusions:
A small library of 2,3-dihydro,3-β-substituted withaferin A analogues was synthesised using Michael addition and Huisgen’s (3+2) cycloaddition reactions and screened to study their cytotoxic potential against various human cancer cell lines. It was observed that the fine tuning in ring A of the α, β-unsaturated group in withaferin A can facilitate the development of the more potent lead molecules. The nature of the substituent at C-3 rather than the α, β-unsaturated carbonyl group is essential for the cytotoxicity.

9. Experimental:

9.1. General methods:
\(^1\)H and \(^13\)C NMR spectra were recorded on 200 and 500 MHz spectrometers with TMS as the internal standard. Chemical shifts are expressed in parts per million (δ ppm); J values are given in Hertz. MS were recorded on Jeol MSD-300 and Bruker Esquire 3000 GC-Mass spectrometer. IR spectra (KBr) discs were recorded on a FT-IR Bruker (270-30) spectrophotometer. Silica gel coated aluminium plates coated on alumina from M/s Merck were used for TLC. Elemental analyses were performed on Elementar Vario EL-III. Optical rotations were measured on Perkin-Elmer 241 polarimeter at 25 °C using sodium D light. MPs were measured in a Buchi-510 apparatus. Reagents and solvents used were mostly of AR grade. The chromatograms were visualised under UV-254-366 nm and ceric ammonium sulphate spray. In vitro cytotoxic studies were performed in collaboration with pharmacological division of IIIM, Jammu.
9.2. Isolation of Withaferin A: Withaferin A was obtained from natural product chemistry division of the parent institute (Indian Institute of Integrative Medicine (IIIM), CSIR-India) and subjected to structural modifications.

9.3. General reaction procedure for Michael type addition:
Triethyl amine was added to a solution of TMSN₃ (1.2 equiv.) in dry methanol (3 mL) at room temperature to maintain the pH of 8.5. Withaferin A (1 equiv., 470 mg, 1 mmol) was separately dissolved in dry methanol (2 mL) and the solution was added to the methanolic solution of nucleophile and kept for the required time. The progress and the completion of the reaction was monitored through TLC. The reaction mixture was dried completely, dissolved in water (5 mL) and extracted with CHCl₃ (10 mL) three times to obtain the product.

9.4. General procedure for Hugien’s (3+2) cycloaddition:
CuI (10 mol %) was added to a solution of 2,3-dihydro,3-β-azidowithaferin A (37) (513 mg, 1 mmol) and alkyne (1 mmol) in acetonitrile. The reaction mixture was stirred for 6-7 h. at room temperature and the completion of the reaction was monitored through TLC. The reaction mixture was filtered and the filtrate was concentrated over rotary evaporator, passed through silica gel column to yield the desired product (51-54).

10.0. Spectral analysis of compounds:
10.1. Prepared by the general procedure (Section 9.3) using 1.8 mmol (878 mg) of 1 and purified on silica gel (EtOAc/PE: 1/1) to obtain the product 37 (73%) as white solid: [α]²⁰/D = +10 (c 0.1, CHCl₃); mp 280-285 °C; IR, 2106 cm⁻¹. ¹H NMR (CDCl₃, 500 MHz): δ 4.40 (dt, J = 3.5, 12.0 Hz, 2H, H-22), 4.30 (brs, 2H, H-27), 4.08 (ddd, J = 3.3, 4.1, 15.7 Hz, 1H, H-3), 3.46 (d, J = 4.1 Hz, 1H, H-4), 3.26 (brs, 1H, H-6), 3.07 (dd, J = 7.3, 15.7 Hz, 2H, H-2), 2.54-2.53 (m, 2H, H-23), 2.35-2.32 (m, 1H), 2.05 (s, 3H, CH₃-28), 1.92-1.90 (m, 3H), 1.67-1.65 (m, 2H), 1.39-1.37 (m, 2H), 1.36-1.34 (m, 2H), 1.24 (s, 3H, CH₃-19), 1.14-1.13 (m, 2H), 1.11-1.10 (m, 2H), 0.93 (d, J = 6.9, 3H, CH₃-21), 0.67 (s, 3H, CH₃-18). ¹³C NMR (CDCl₃, 50 MHz): δ 208.5 (qC, C-1), 38.75 (CH₂, C-2), 58.8 (CH, C-3), 75.1 (CH, C-4), 65.1 (qC, C-5), 59.8 (CH, C-6), 29.8 (CH₂, C-7), 29.7 (CH, C-8), 44.3 (CH, C-9), 50.6 (qC, C-10), 21.4 (CH₂, C-11), 27.0 (CH₂, C-12), 42.7 (qC, C-13), 56.1 (CH, C-14), 24.4 (CH₂, C-15), 39.1 (CH₂, C-16), 51.1 (CH, C-17), 9.9 (CH₃, C-18), 14.8 (CH₃, C-19), 39.0 (CH, C-20), 13.1 (CH₃, C-21), 78.0 (CH, C-22), 29.6 (CH₂, C-23), 125.7 (qC, C-24), 153.2 (qC, C-25), 167.8 (qC, C-26), 55.3 (CH₂, C-27), 20.0 (CH₃, C-
Chapter 4

28); ESI-MS (m/z): 536 [M+Na]⁺; Anal. Calc. for C₂₈H₃₉N₃O₆: C, 65.47, H, 7.67, N, 8.18; Found: C, 65.51, H, 7.60, N, 8.10.

10.2. Prepared by the general procedure (Section 9.3) using 1mmol (470 mg) of 1 and purified on silica gel (EtOAc/PE: 45/55) to obtain the product 38 (85%) as white solid: [α]D₂⁰ = +10 (c 0.1, CHCl₃); mp 264-269 °C; ¹H NMR (CDCl₃, 500 MHz): δ 7.50 (d, J = 7.5 Hz, 2H, Ar), 7.32 (d, J = 7.2 Hz, 2H, Ar), 7.24-7.21 (m, 1H, Ar), 4.40 (dt, J = 5.5, 12.0 Hz, 2H, H-22), 4.29 (brs, 2H, H-27), 3.45, (bd, J = 3.8 Hz, 1H, H-4), 3.74-3.68 (m, 1H, H-3), 3.22 (bs, 1H, H-6), 2.56 (dd, J = 4.5, 11.7 Hz, 2H, H-2), 2.50-2.48 (m, 2H, H-23), 2.35-2.33 (m, 1H), 1.99 (s, 3H, H-28), 1.94-1.91 (m, 3H), 1.65-1.63 (m, 2H), 1.40-1.38 (m, 2H), 1.35-1.33 (m, 2H), 1.27 (s, 3H, CH₃-19), 1.16-1.14 (m, 2H), 1.11-1.09 (m, 2H), 1.02 (d, J = 6.9 Hz, 3H, H-21), 0.64 (s, 3H, CH₃-18). ¹³C NMR (CDCl₃, 50 MHz): δ 208.0 (qC, C-1), 28.4 (CH₂, C-2), 42.1 (CH, C-3), 70.0 (CH, C-4), 65.1 (qC, C-5), 59.3 (CH, C-6), 29.7 (CH₂, C-7), 29.8 (CH, C-8), 44.1 (CH, C-9), 50.4 (qC, C-10), 21.5 (CH₂, C-11), 27.0 (CH₂, C-12), 42.6 (qC, C-13), 56.0 (CH, C-14), 24.2 (CH₂, C-15), 39.0 (CH₂, C-16), 51.9 (CH, C-17), 10.5 (CH₃, C-18), 14.6 (CH₃, C-19), 39.0 (CH, C-20), 13.3 (CH₃, C-21), 77.7 (CH, C-22), 29.6 (CH₂, C-23), 125.6 (qC, C-24), 153.9 (qC, C-25), 167.6 (qC, C-26), 55.7 (CH₂, C-27), 19.0 (CH₃, C-28), 132.0 (C-Ar), 128.4 (CH-Ar), 128.3 (CH-Ar), 128.2 (CH-Ar), 126.5 (CH-Ar), 126.4 (CH-Ar); ESI-MS (m/z): 603 [M+Na]⁺; Anal. Calc. for C₃₄H₄₄O₈S: 70.31, H, 7. 64; Found: C, 70.39, H, 7.70.

10.3. Prepared by the general procedure (Section 9.3) using 1 mmol (470 mg) of 1 and purified on silica gel (EtOAc/PE: 45/55) to obtain the product 39 (82%) as white solid: [α]D₂⁰ = +10 (c 0.1, CHCl₃); mp 290-294 °C; ¹H NMR (CDCl₃, 500 MHz): δ 7.46 (d, J = 8.1 Hz, 2H, Ar), 7.12 (d, J = 8.3 Hz, 2H, Ar), 4.41 (dt, J = 5.5, 12.0 Hz, 2H, H-22), 4.27 (brs, 2H, H-27), 3.72-3.69 (m, 1H, H-3), 3.47 (d, J = 4.4 Hz, 1H, H-4), 3.23 (bs, 1H, H-6), 2.45 (dd, J = 4.3, 10.9 Hz, 2H, H-2), 2.43-2.40 (m, 2H), 2.35-2.32 (m, 1H), 2.01 (s, 3H, H-28), 1.95-1.94 (m, 3H), 1.68-1.66 (m, 2H), 1.39-1.37 (m, 2H), 1.36-1.34 (m, 2H), 1.21 (s, 3H, CH₃-19), 1.15-1.14 (m, 2H), 1.12-1.10 (m, 2H), 1.01 (d, J = 6.9 Hz, 3H, H-21), 0.60 (s, 3H, CH₃-18). ¹³C NMR (CDCl₃, 50MHz): 207.9 (qC, C-1), 29.1 (CH₂, C-2), 43.2 (CH, C-3), 75.6 (CH, C-4), 65.1 (qC, C-5), 60.4 (CH, C-6), 29.7 (CH₂, C-7), 29.7 (CH, C-8), 44.1 (CH, C-9), 50.5 (qC, C-10), 21.5 (CH₂, C-11), 27.5 (CH₂, C-12), 42.7 (qC, C-13), 56.0 (CH, C-14), 24.2 (CH₂, C-15), 39.0
(CH₂, C-16), 51.9 (CH, C-17), 9.6 (CH₂, C-18), 14.2 (CH₃, C-19), 39.0 (CH, C-20), 13.7 (CH₃, C-21), 77.7 (CH, C-22), 29.6 (CH₂, C-23), 125.6 (qC, C-24), 153.1 (qC, C-25), 167.6 (qC, C-26), 55.7 (CH₂, C-27), 20.0 (CH₃, C-28), 134.2 (qC, Ar), 132.6 (qC, Ar), 128.9, 128.8, 128.3, 128.2 (4 x CH, Ar); ESI-MS (m/z): 638 [M+Na]⁺; Anal. Calc. for C₃₅H₄₃ClO₆S: C, 66.45, H, 7.04; Found: C, 66.39, H, 7.11.

10.4. Prepared by the general procedure (Section 9.3) using 1 mmol (470 mg) of compound 1 and purified on silica gel (EtOAc/PE: 43/57) to obtain the product 41 (85%) as white solid: [α]²⁰_D = +10 (c 0.1, CHCl₃); mp 240-245 ºC; ¹H NMR (CDCl₃, 500 MHz): δ 7.44 (d, J = 8.5 Hz, 2H, Ar), 6.8 (d, J = 8.3 Hz, 2H, Ar), 4.39 (dt, J = 5.5, 12.0 Hz, 2H, H-22), 4.26 (brs, 2H, H-27), 3.83 (s, 3H, OMe-Ar), 3.68-3.66 (m, 1H, H-4), 3.43 (d, J = 4.2 Hz, 1H, H-3), 3.21 (bs, 1H, H-6), 2.52, (dd, J = 4.2, 11.3 Hz, 2H, H-2), 2.43-2.41 (m, 2H), 2.35-2.33 (m, 1H), 2.06 (bs, 3H, H-28), 1.98-1.96 (m, 3H), 1.65-1.63 (m, 2H), 1.37-1.36 (m, 2H), 1.34-1.33 (m, 2H), 1.28 (s, 3H, CH₃-19), 1.16-1.14 (m, 2H), 1.12-1.10 (m, 2H), 0.99 (d, J = 6.6 Hz, 3H, CH₃-21), 0.61 (s, 3H, CH₃-18). ¹³C NMR (CDCl₃, 50 MHz): δ 209.6 (qC, C-1), 29.7 (CH₂, C-2), 41.1 (CH, C-3), 75.8 (CH, C-4), 65.1 (qC, C-5), 59.5 (CH, C-6), 29.6 (CH₂, C-7), 29.4 (CH, C-8), 44.1 (CH, C-9), 50.3 (qC, C-10), 21.3 (CH₂, C-11), 26.9 (CH₂, C-12), 42.6 (qC, C-13), 56.9 (CH, C-14), 24.0 (CH₂, C-15), 39.1 (CH₂, C-16), 51.9 (CH, C-17), 10.5 (CH₃, C-18), 14.7 (CH₃, C-19), 39.0 (CH, C-20), 13.1 (CH₃, C-21), 78.3 (CH, C-22), 29.7 (CH₂, C-23), 125.8 (qC, C-24), 153.5 (qC, C-25), 167.7 (qC, C-26), 56.7 (CH₂, C-27), 19.6 (CH₃, C-28), 159.9 (qC, Ar), 132.6 (qC, Ar), 114.7, 114.6, 128.4, 128.3, 128.2 (5 x CH, Ar), 55.3 (OMe, Ar); ESI-MS (m/z): 633 [M+Na]⁺; Anal. Calc. for C₃₅H₄₃ClO₆S: C, 68.82, H, 7.59; Found: 68.95, H, 7.67.

10.5. Prepared by the general procedure (Section 9.3) using 1 mmol (470 mg) of compound 1 and purified on silica gel (EtOAc/PE: 45/55) to obtain the product 42 (84%) as white solid: [α]²⁰_D = +0 (c 0.1, CHCl₃); mp 284-289 ºC; ¹H NMR (CDCl₃, 500 MHz): δ 7.32 (d, J = 9.3Hz, 1H, H-6'), 6.31-6.20 (m, 2H, H-4', H-5'), 4.4 (dt, J = 5.5, 12.0 Hz, 2H - 22), 4.28 (brs, 2H, H-27), 3.71 (bs, 1H, H-4), 3.58 (d, J = 13.1 Hz, 1H, H-1'a/b), 3.48 (d, J = 13.1 Hz, 1H, H-1'a/b), 3.45-3.43 (m, 1H, H-3), 3.22 (bs, 1H, H-6), 2.54-2.53 (m, 2H), 2.51 (dd, J = 4.5, 11.0 Hz, 2H, H-2), 2.48-2.44 (m, 2H, H-2'), 2.35 (m, 1H), 2.00 (s, 3H, CH₃-28), 1.94-1.93 (m, 3H), 1.69-1.67 (m, 2H), 1.41-
1.39 (m, 2H), 1.35-1.33 (m, 2H), 1.26 (s, 3H, CH−3−19), 1.14-1.13 (m, 2H), 1.12-1.11 (m, 2H), 1.02 (d, J = 7.0 Hz, 3H, CH−21), 0.62 (s, 3H, CH−3−18), 13C NMR (CDCl3, 50 MHz): δ 209.8 (qC, C-1), 128.3, 128.2 (5x CH, Ar), 63.4 (CH, C-2), 42.2 (CH, C-3), 75.4 (CH, C-4), 65.3 (qC, C-5), 60.0 (CH, C-6), 29.7 (CH, C-7), 29.7 (CH, C-8), 44.0 (CH, C-9), 50.3 (qC, C-10), 22.1 (CH2, C-11), 27.3 (CH2, C-12), 42.8 (qC, C-13), 56.1 (CH, C-14), 24.8 (CH2, C-15), 39.3 (CH2, C-16), 51.9 (CH, C-17), 10.3 (CH3, C-18), 14.5 (CH3, C-19), 39.8 (CH, C-20), 13.6 (CH3, C-21), 78.9 (CH, C-22), 29.7 (CH2, C-23), 125.9 (qC, C-24), 153.0 (qC, C-25), 167.9 (qC, C-26), 56.1 (CH2, C-27), 20.2 (CH3, C-28), 151.1 (qC, C-3’), 142.2 (CH, C-6’), 110.6 (CH, C-5’), 107.9 (CH, C-4’), 31.1 (CH2, C-1’), 30.9 (CH2, C-2’); ESI-MS (m/z): 607 [M+Na]+; Anal. Cal. for C33H44O7S: C, 67.78, H, 7.58; Found: 67.86, H, 7.47.

10.6. Prepared by the general procedure (Section 9.3) using 1 mmol (470 mg) of compound 1 and purified on silica gel (EtOAc/PE: 60/40) to obtain the product 43 (78%) as white solid: [α]D20 = +10 (c 0.1, CHCl3); mp 233-238 °C; 1H NMR (CDCl3, 500 MHz): δ 7.46-7.25 (m, 5H, Ar-H), 4.41 (dt, J = 5.5, 11.9 Hz, 2H, H-22), 4.27 (s, 2H, H-27), 3.71 (bs, 3H, -COOCH3), 3.60 (dd, J = 5.0, 10.7 Hz, 1H, H-2’), 3.30 (d, J = 3.1 Hz, 1H, H-4), 3.10 (bs, 1H, H-6), 3.03-3.01 (m, 1H, H-3), 3.01 (d, J = 5.1 Hz, 2H, H-3’), 2.67 (bd, J = 14.7 Hz, 2H, H-2), 2.54-2.52 (m, 2H), 2.35-2.32 (m, 1H), 2.03 (bs, 3H, CH3-28), 1.98-1.96 (m, 3H), 1.70-1.68 (m, 2H), 1.42-1.40 (m, 2H), 1.39-1.38 (m, 2H), 1.28 (s, 3H, CH3-19), 1.17-1.15 (m, 2H), 1.13-1.12 (m, 2H), 0.98 (d, J = 6.1 Hz, 3H, CH3-21), 0.68 (s, 3H, CH3-18), 13C NMR (CDCl3, 50 MHz): δ 209.5 (qC, C-1), 39.7 (CH2, C-2), 42.9 (CH, C-3), 76.5 (CH, C-4), 65.1 (qC, C-5), 61.8 (CH, C-6), 31.1 (CH2, C-7), 29.6 (CH, C-8), 44.4 (CH, C-9), 50.4 (qC, C-10), 21.2 (CH2, C-11), 27.6 (CH2, C-12), 42.5 (qC, C-13), 56.4 (CH, C-14), 23.9 (CH2, C-15), 39.2 (CH2, C-16), 51.9 (CH, C-17), 11.6 (CH3, C-18), 17.0 (CH3, C-19), 39.2 (CH, C-20), 13.3 (CH3, C-21), 78.9 (CH, C-22), 29.6 (CH2, C-23), 125.2 (qC, C-24), 153.5 (qC, C-25), 167.6 (qC, C-26), 56.9 (CH2, C-27), 20.0 (CH3, C-28), 175.2 (qC, -COOMe), 138.1 (qC, Ar), 63.4 (CH, C-2’), 38.6 (CH2, C-2’), 126.3, 128.9, 128.4, 128.3, 128.2 (5x CH, Ar); ESI-MS (m/z): 672 [M+Na]+; Anal. Cal. for C38H51NO6: C, 70.29, H, 7.91, N, 2.16; Found: 70.31, H, 7.99, N, 2.21.
10.7. Prepared by the general procedure (Section 9.3) using 1 mmol (470 mg) of compound 1 and purified on silica gel (EtOAc/PE: 60/40) to obtain the product 44 (81%) as white solid: $[\alpha]^{20}_D = +10 \, (c \, 0.1, \text{CHCl}_3)$; mp 220-225 °C; $^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 4.40 (dt, $J = 5.5, 12.0$ Hz, 2H, H-22), 4.26 (brs, 2H, H-27), 3.76 (bs, 3H, - COOCH$_3$), 3.40 (d, $J = 3.4$ Hz, 1H, H-4), 3.31 (bs, 1H, H-6), 3.14 (d, $J = 4.4$ Hz, 1H, H-1’), 3.05 (dd, $J = 5.1, 14.7$ Hz, 1H, H-3), 3.02 (bd, $J = 6.5$ Hz, 1H, H-3’), 2.54-2.51 (m, 2H), 2.35-2.34 (m, 1H), 2.32 (dd, $J = 4.1, 14.1$ Hz, 2H, H-2), 2.00 (bs, 3H, CH$_3$-28), 1.96-1.95 (m, 3H), 1.83-1.81 (m, 1H, H-4’), 1.69-1.68 (m, 2H), 1.65-1.62 (m, 2H, H-2’), 1.39-1.38 (m, 2H), 1.37-1.35(m, 2H), 1.23 (s, 3H, CH$_3$-19), 1.15-1.13 (m, 2H), 1.10-1.08 (m, 2H), 0.99 (d, $J = 6.5$ Hz, 3H, CH$_3$-18), 0.90 (d, $J = 6.5$ Hz, 3H, CH$_3$), 0.88 (d, $J = 6.5$ Hz, 3H, CH$_3$), 0.69 (s, 3H, CH$_3$-21), $^{13}$C NMR (CDCl$_3$, 50 MHz): $\delta$ 210.1 (qC, C-1), 37.7 (CH$_2$, C-2), 42.9 (CH, C-3), 78.7 (CH, C-4), 65.4 (qC, C-5), 63.0 (CH, C-6), 30.9 (CH$_2$, C-7), 28.9 (CH, C-8), 44.3 (CH, C-9), 50.4 (qC, C-10), 21.0 (CH$_2$, C-11), 27.2 (CH$_2$, C-12), 42.0 (qC, C-13), 56.8 (CH, C-14), 24.8 (CH$_2$, C-15), 39.1 (CH$_2$, C-16), 51.7 (CH, C-17), 10.5 (CH$_3$, C-18), 14.2 (CH$_3$, C-19), 39.5 (CH, C-20), 13.9 (CH$_3$, C-21), 75.5 (CH, C-22), 29.6 (CH$_2$, C-23), 125.9 (qC, C-24), 153.0 (qC, C-25), 167.1 (qC, C-26), 55.7 (CH$_2$, C-27), 20.3 (CH$_3$, C-28), 175.4 (qC, COOCH$_3$), 61.3 (CH, C-2’), 55.7 (CH$_3$, OMe), 44.2 (CH$_2$, C-3’), 24.1 (CH, C-4’), 22.6, 22.5 (2 x CH$_3$); ESI-MS (m/z): 624 [M+Na]$^+$; Anal. Calc. for C$_{34}$H$_{51}$NO$_6$: C, 67.86, H, 8.54, N, 2.33; Found: 67.80, H, 8.63, N, 2.39.

10.8. Prepared by the general procedure (Section 9.3) using 1 mmol (470 mg) of compound 1 and purified on silica gel (EtOAc/PE: 60/40) to obtain the product 45 (83%) as white solid: $[\alpha]^{20}_D = +10 \, (c \, 0.1, \text{CHCl}_3)$; mp 257-260 °C; $^1$H NMR (CDCl$_3$, 500 MHz): $\delta$ 4.40 (dt, $J = 5.5, 12.0$ Hz, 2H, H-22), 4.29 (brs, 2H, H-27), 3.80 (s, 3H, - COOCH$_3$), 3.79 (dd, $J = 4.1, 15.2$ Hz, 1H, H-3’), 3.4 (d, $J = 4.0$ Hz, 1H, H-4), 3.35 (d, $J = 4.1$ Hz, 2H, H-2’), 3.23 (bs, 1H, H-6), 3.0 (ddd, $J = 4.5, 5.1, 6.4$ Hz, 1H, H-3), 2.57-2.55 (m, 1H, H-1’), 2.54-2.52 (m, 2H), 2.35 (dd, $J = 4.5, 11.7$ Hz, 2H, H-2), 2.29-2.26 (m, 1H), 1.99 (bs, 3H, CH$_3$-28), 1.95-1.93 (m, 3H), 1.70-1.68 (m, 2H), 1.43-1.41 (m, 2H), 1.38-1.37 (m, 2H), 1.26 (s, 3H, CH$_3$-19), 1.16-1.15 (m, 2H), 1.10-1.08 (m, 2H), 0.98 (d, $J$
Chapter 4

= 6.5 Hz, 3H), 0.69 (s, 3H, CH₃-21), ¹³C NMR (CDCl₃, 50 MHz): δ 209.8 (qC, C-1), 39.9 (CH₂, C-2), 43.2 (CH, C-3), 76.5 (CH, C-4), 65.1 (qC, C-5), 61.8 (CH, C-6), 31.1 (CH₂, C-7), 29.9 (CH, C-8), 44.2 (CH, C-9), 49.5 (qC, C-10), 21.6 (CH₂, C-11), 27.7 (CH₂, C-12), 42.6 (qC, C-13), 56.0 (CH, C-14), 24.4 (CH₂, C-15), 39.0 (CH₂, C-16), 51.9 (CH, C-17), 11.6 (CH₃, C-18), 14.4 (CH₃, C-19), 39.0 (CH, C-20), 13.6 (CH₃, C-21), 78.9 (CH, C-22), 29.7 (CH₂, C-23), 125.6 (qC, C-24), 153.9 (qC, C-25), 167.7 (qC, C-26), 56.8 (CH₂, C-27), 20.6 (CH₃, C-28), 172.9 (qC, COOMe), 54.0 (CH₃, OMe), 60.1 (CH, C-3;), 37.8 (CH₂, C-2") ; ESI-MS (m/z): 628 [M+Na]⁺; Anal. Calc. for C₃₂H₄₇NO₈S: C, 63.45, H, 7.82, N, 2.31; Found: 63.52, H, 7.90, N, 2.39.

10.9. Prepared by the general procedure (Section 9.4) using 1mmol (513 mg) of compound 1 and purified on silica gel (EtOAc/PE: 65/35) to obtain the product 50 (99%) as white solid: [α]²⁰ d = +10 (c 0.1, CHCl₃); mp 310-315 °C; ¹H NMR (CDCl₃, 500 MHz): δ 8.6 (bs, 1H, Triazole), 4.40 (dt, J = 5.5, 12.0 Hz, 2H, H-22), 4.29 (brs, 2H, H-27), 3.9 (bs, 3H, CO₂Me), 3.72 (dd, J = 4.5, 16.5 Hz, 1H, H-4), 3.43 (d, J = 3.0 Hz, 1H, H-3), 3.20 (bs, 1H, H-6), 3.11 (dd, J = 9.6, 16.5 Hz, 2H, H-2), 2.54-2.52 (m, 2H), 2.35-2.33 (m, 1H), 2.10 (s, 3H, CH₃-28), 1.99-1.97 (m, 3H), 1.67-1.65 (m, 2H), 1.37-1.35 (m, 2H), 1.34-1.32 (m, 2H), 1.27 (s, 3H, CH₃-19), 1.14-1.12 (m, 2H), 1.11-1.10 (m, 2H), 1.02 (d, J = 6.7 Hz, 3H, CH₃-21), 1.31 (s, 3H, CH₃-19), 0.72 (bs,3H, CH₃-18), ¹³C NMR (CDCl₃, 50 MHz):δ 209.5 (qC, C-1), 39.8 (CH₂, C-2), 57.9 (CH, C-3), 65.1 (qC, C-5), 76.5 (CH, C-4), 59.4 (CH, C-6), 32.0 (CH₂, C-7), 30.1 (CH, C-8), 43.9 (CH, C-9), 50.5 (qC, C-10), 22.9 (CH₂, C-11), 27.9 (CH₂, C-12), 42.0 (qC, C-13), 56.0 (CH, C-14), 24.9 (CH₂, C-15), 39.2 (CH₂, C-16), 51.9 (CH, C-17), 11.6 (CH₃, C-18), 17.0 (CH₃, C-19), 39.0 (CH, C-20), 13.3 (CH₃, C-21), 78.7 (CH, C-22), 29.9 (CH₂, C-23), 125.0 (qC, C-24), 156.5 (qC, C-25), 167.9 (qC, C-26), 56.9 (CH₂, C-27), 20.1 (CH₃, C-28), 160.8 (qC, COOMe), 139.4 (qC, triazole), 128.9 (CH, triazole), 55.8 (CH₃, COOMe); ESI-MS (m/z): 620 [M+Na]⁺; Anal. Calc. for C₃₂H₄₇N₃O₈: C, 64.30, H, 7.25, N, 7.03; Found: 64.38, H, 7.33, N, 7.11.

10.10. Prepared by the general procedure (Section 9.4) using 1mmol (513 mg) of compound 37 and purified on silica gel (EtOAc/PE: 70/30) to obtain the product 51 (99%) as white solid: [α]²⁰ d = +10 (c 0.1, CHCl₃); mp 290-295 °C; ¹H NMR (CDCl₃, 500
Chapter 4

Prepared by the general procedure (Section 9.4) using 1mmol (513 mg) of compound 37 and purified on silica gel (EtOAc/PE: 72/28) to obtain the product 52 (99%) as white solid: [α]D = +10 (c 0.1, CHCl3); mp 284-289 °C; ¹H NMR (CDCl3, 500 MHz): δ 9.86 (bs, 1H, -CHO), 7.66 (bs, 1H, triazole), 7.44 (d, J = 7.0 Hz 2H, Ar), 7.16 (d, J = 8.1 Hz, 1H, Ar), 4.99 (bs, 2H), 4.38 (dt, J = 5.5, 12.0 Hz, 2H, H-22), 4.26 (brs, 2H, H-27), 3.84 (s, 3H, OMe-Ar), 3.71 (dd, J = 4.6, 16.2 Hz, 1H, H-4), 3.40 (d, J = 4.2 Hz, 1H, H-3), 3.31 (bs, 1H, H-6), 2.55-2.54 (dd, J = 4.5, 11.7 Hz, 1H), 2.51-2.50 (m, 2H), 2.34-2.33 (m, 1H), 2.03 (bs, 3H, CH3-28), 1.98-1.96 (m, 3H), 1.70-1.68 (m, 2H), 1.42-1.40 (m, 2H), 1.39-1.38 (m, 2H), 1.28 (s, 3H, CH3-19), 1.17-1.15 (m, 2H), 1.13-1.12 (m, 2H), 0.98 (d, J = 6.1 Hz, 3H, CH3-21), 0.68 (s, 3H, CH3-18), ¹³C NMR (CDCl3, 500 MHz): δ 210.1 (qC, C-1), 37.6 (CH2, C-2), 58.8 (CH, C-3), 78.7 (CH, C-4), 65.0 (qC, C-5), 59.9 (CH, C-6), 30.9 (CH2, C-7), 29.9 (CH, C-8), 45.0 (CH, C-9), 50.6 (qC, C-10), 22.0 (CH2, C-11), 27.2 (CH2, C-12), 42.9 (qC, C-13), 56.7 (CH, C-14), 24.7 (CH2, C-15), 39.2 (CH2, C-16), 51.7 (CH, C-17), 10.5 (CH3, C-18), 14.1 (CH3, C-19), 38.9 (CH, C-20), 13.9 (CH3, C-21), 75.5 (CH, C-22), 29.7 (CH2, C-23), 125.7 (qC, C-25), 153.7 (qC, C-24), 167.9 (qC, C-26), 55.7 (CH2, C-27), 20.3 (CH3, C-28), 199.0 (qC, CO), 153.1, 115.9, 115.8, 114.4, 114.3 (4 x CH, Ar), 127.8 (CH, Triazole), 70.4 (CH2, OCH2); ESI-MS (m/z): 698 [M+Na]⁺; Anal. Calc. for C38H49N3O8: C, 67.53, H, 7.31, N, 6.22; Found: 67.62, H, 7.39, N, 6.29.
152.2, 122.5 (3 x q, C, Ar), 139.2 (CH, triazole), 123.2, 121.8, 118.3, 117.9 (4 x CH, Ar), 70.9 (-OCH2); ESI-MS (m/z): 603 [M+Na]+; Anal. Calc. for C39H49N3O9: C, 66.55, H, 7.02, N, 5.97; Found: 66.63, H, 7.12, N, 5.90.

10.12. Prepared by the general procedure (Section 9.4) using 1 mmol (513 mg) of compound 37 and purified on silica gel (EtOAc/PE: 75/25) to obtain the product 53 (73%) as white solid:

\([\alpha]^{20}_{D} = +10 (c 0.1, \text{CHCl}_3)\); mp 220-225 °C; \(^1\text{H} \text{NMR (CDCl}_3\), 500 MHz): \(\delta 7.7 (\text{bs, 1H, triazole}), 5.87 (d, J = 3.7 \text{Hz, 1H, H-1'}), 4.83 (\text{bs, 2H, -OCH}_2), 4.59 (d, J = 3.7 \text{Hz, 1H}), 4.42 (dt, J = 5.5, 12.0 \text{Hz, H-22}), 4.30-4.29 (m, 2H), 4.28 (brs, 2H, H-27), 4.10-4.07 (m, 2H), 4.01 (dd, J = 5.3, 8.2 \text{Hz, 1H}), 3.65 (dd, J = 5.4, 15.9 \text{Hz, 1H, H-4}), 3.46 (d, J = 4.1 \text{Hz, 1H, H-3}), 3.26 (bs, 1H, H-6), 2.55-2.54 (dd, J = 4.5, 11.7 \text{Hz, 2H, H-2}), 2.52-2.50 (m, 2H), 2.32-2.30 (m, 1H), 2.00 (bs, 3H, CH3-28), 1.95-1.93 (m, 3H), 1.71-1.69 (m, 2H), 1.49 (s, 3H, CH3), 1.40 (s, 3H, CH3), 1.39-1.38 (m, 2H), 1.37-1.36 (m, 2H), 1.34 (s, 3H, CH3), 1.31 (s, 3H, CH3), 1.26 (s, 3H, CH3-19), 1.15-1.14 (m, 2H), 1.12-1.11 (m, 2H), 0.98 (d, J = 6.1 \text{Hz, 3H, CH3-21}), 0.68 (s, 3H, CH3-18), 0.66 (s, 3H), 0.99 (d, J = 6.5 \text{Hz, 3H, CH3-21}), \(^1\text{C} \text{NMR (CDCl}_3\), 50 MHz): \(\delta 209.5 (\text{qC, C-1}), 38.9 (\text{CH}_2, C-2), 58.7 (\text{CH, C-3}), 78.7 (\text{CH, C-4}), 65.9 (\text{qC, C-5}), 60.0 (\text{CH, C-6}), 30.5 (\text{CH}_2, C-7), 29.9 (\text{CH, C-8}), 43.9 (\text{CH, C-9}), 49.9 (\text{qC, C-10}), 21.9 (\text{CH}_2, C-11), 27.9 (\text{CH}_2, C-12), 42.7 (\text{qC, C-13}), 56.9 (\text{CH, C-14}), 24.5 (\text{CH}_2, C-15), 39.7 (\text{CH}_2, C-16), 51.9 (\text{CH, C-17}), 11.5 (\text{CH}_3, C-18), 15.3 (\text{CH}_3, C-19), 39.0 (\text{CH, C-20}), 13.3 (\text{CH}_3, C-21), 76.7 (\text{CH, C-22}), 29.5 (\text{CH}_2, C-23), 153.9 (\text{qC, C-24}), 125.8 (\text{qC, C-25}), 167.9 (\text{qC, C-26}), 55.7 (\text{CH}_2, C-27), 20.9 (\text{CH}_3, C-28); ESI-MS (m/z): 834 [M+Na]+; Anal. Calc. for C43H61N3O12: C, 63.61, H, 7.57, N, 5.18; Found: C, 63.69, H, 7.64, N, 5.26.

10.13. Prepared by the general procedure (Section 9.4) using 1 mmol (513 mg) of compound 37 and purified on silica gel (EtOAc/PE: 80/20) to obtain the product 54 (99%) as white solid:

\([\alpha]^{20}_{D} = +10 (c 0.1, \text{CHCl}_3)\); mp 240-245 °C; \(^1\text{H} \text{NMR (CDCl}_3\), 500 MHz): \(\delta 7.64 (\text{bs, 1H}, 5.34 (d, J = 5.4 \text{Hz, 1H, H-1'}), 5.16 (dd, J = 8.0, 10.0 \text{Hz, 1H, H-4'}), 4.98 (dd, J = 3.4, 10.0 \text{Hz, 1H, H-2'}), 4.90 (bs, 2H, OCH2), 4.59 (t, J = 8.0, 17.2 \text{Hz, 1H, H-3'}), 4.41 (dt, J = 5.5, 12.0 \text{Hz, H-22}), 4.25 (bs, 2H, H-27), 4.14 (dd, J = 6.5, 11.0 \text{Hz, 1H, H-6a/H-6b}), 4.07-4.04 (m, 1H, H-6a/H-6b), 3.91 (m, 1H, H-5'), 3.44 (d, J = 5.0 \text{Hz, 1H, H-4}), 3.25 (bs, 1H, H-6), 3.11 (dd, J = 9.2, 16.0 \text{Hz, 1H, H-3}), 2.57-2.55 (dd, J = 4.5, 9.2 \text{Hz, 1H}), 2.54-2.52 (m, 2H), 2.35-2.31
Chapter 4

(\text{m}, 1\text{H}), 2.09 (s, 3\text{H}, -\text{OAc}), 2.05 (s, 3\text{H}, \text{CH}_3-28), 1.99 (s, 3\text{H}, -\text{OAc}), 1.98 (s, 3\text{H}, -\text{OAc}), 1.96 (s, 3\text{H}, -\text{OAc}), 1.95-1.94 (m, 3\text{H}), 1.67-1.66 (m, 2\text{H}), 1.42-1.40 (m, 2\text{H}), 1.39-1.38 (m, 2\text{H}), 1.28 (s, 3\text{H}, \text{CH}_3-19), 1.17-1.15 (m, 2\text{H}), 1.13-1.12 (m, 2\text{H}), 0.98 (d, J = 6.1 \text{ Hz}, 3\text{H}, \text{CH}_3-21), 0.68 (s, 3\text{H}, \text{CH}_3-18), ^{13}\text{C} \text{ NMR} (\text{CDCl}_3, 50 \text{ MHz}) \; \delta \; 208.9 \; (\text{qC}, \text{ C}-1), 39.7 \; (\text{CH}_2, C-2), 57.3 \; (\text{CH}, C-3), 76.1 \; (\text{CH}, C-4), 64.9 \; (\text{qC}, C-5), 61.2 \; (\text{CH}, C-6), 31.9 \; (\text{CH}_2, C-7), 29.9 \; (\text{CH}, C-8), 44.3 \; (\text{CH}, C-9), 50.6 \; (\text{qC}, C-10), 21.8 \; (\text{CH}_2, C-11), 27.9 \; (\text{CH}_2, C-12), 42.9 \; (\text{qC}, C-13), 55.9 \; (\text{CH}, C-14), 24.6 \; (\text{CH}_2, C-15), 39.9 \; (\text{CH}_2, C-16), 52.0 \; (\text{CH}, C-17), 11.5 \; (\text{CH}_3, C-18), 14.1 \; (\text{CH}_3, C-19), 38.9 \; (\text{CH}, C-20), 13.1 \; (\text{CH}_3, C-21), 79.6 \; (\text{CH}, C-22), 29.6 \; (\text{CH}_2, C-23), 153.3 \; (\text{qC}, C-24), 124.7 \; (\text{qC}, C-25), 167.9 \; (\text{qC}, C-26), 55.6 \; (\text{CH}_2, C-27), 20.4 \; (\text{CH}_3, C-28), 110.5, 70.7, 70.6, 68.6, 66.8 \; (5 \times \text{CH}, \text{C}-1', \text{C}-2', \text{C}-3', \text{C}-4', \text{C}-5’), 62.0 \; (\text{CH}_2, C-6’), 61.0 \; (\text{CH}_2, -\text{OCH}_2), 170.2, 170.1, 169.6, 167.1 \; (4 \times \text{OAc}), 139.2 \; (\text{CH}, \text{triazole}), 125.5 \; (\text{qC}, 25); \text{ESI-MS} \; (m/z): 922 \; [\text{M}+\text{Na}]^+; \text{Anal. Calc. for C}_{45}\text{H}_{61}\text{N}_3\text{O}_{16}: \text{C}, 60.06, \text{H}, 6.83, \text{N}, 4.67; \text{Found: C}, 60.11, \text{H}, 6.92, \text{N}, 4.73.
$^1$H and $^{13}$C NMR of compound 37:
$^1$H-$^1$H COSY spectra of compound 37:
Chapter 4

HSQC spectra of compound 37:

![HSQC spectra of compound 37](image_url)
HMBC spectra of compound 37:
$^1$H and $^{13}$C NMR of compound 44:
$^1$H and $^{13}$C NMR of compound 43:
$^1$H and $^{13}$C NMR of compound 54: