Identification of Inhibitors of DNA Topoisomerase II from a Synthetic Library of Glycoconjugates

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Abstract: A library of 24 glycoconjugates related to glycosylated β-amino acid derivative (**I**) was been prepared and screened against DNA topoisomerase-II of the filarial parasite *S. cervi*. Among these, compound **6** was found to be a potent inhibitor of DNA topoisomerase-II with 95% inhibition at 1.09μM. Furthermore, compound **6** was at least three times more potent than the lead compound, glycosylated β-amino acid derivative **I**.

The effective treatment regimens to decrease microfilaramia have been primarily responsible for the recent designation by the World Health Assembly of lymphatic filariasis as a disease that can be eliminated globally as a public health problem [1]. Human lymphatic filariasis, which is caused by helminths *Wuchereria bancrofti* (90% cases) and *Brugia malayi* (10% cases), affects approximately 120 million people, with one billion people considered to be at risk of becoming infected [2]. It has been found in 76 countries through the regions of South and Central America, Central Africa, Eastern Mediterranean, Southeast Asia and Western Pacific. The challenge of drug discovery lies in the identification of novel therapeutic targets from the myriad of parasite enzymes, receptors, genome data and metabolic pathways [3]. The potential antifilarials known today either acts on the membrane receptors or on metabolic enzymes. DNA topoisomerases are cellular enzymes that are intricately involved in maintaining the topographic structure of DNA, transcription and mitosis [4]. Topoisomerase has been identified as an important biochemical target in cancer chemotherapy and microbial infections.

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Intracellular bacteria have been detected [5] in most filarial worms, and it is contemplated that by eradicating the endobacteria, filarial parasites may also die due to disturbance in the endosymbiosis. In fact, tetracyclin therapy not only reduces the number of *Walbachia* in filarial oocytes and embryos but also inhibits the filarial embryogenesis [6]. Further we have recently identified DNA topoisomerase II (topo II) of the filarial parasites as a target for the development of antifilarial compounds and hence studied the effect of various inhibitors of topoisomerases and antifilarials. Only nalidixic acid and novobiocin were found to be strong inhibitors of topo II activity of S. *cervi* [7].

A number of other compounds belonging to different classes have been also identified as inhibitors of DNA topoisomerases [8-10] including the established classes quinolones, coumarins and m-AMSA (4'-(9-acridinyl amino) methansulfon-m-anisidine), and the glycine rich peptide Microcin B17. Further, a number of sugar derivatives with aglycon pharmacophores have been reported to inhibit DNA topoisomerases, however, carbohydrates as such have not been studied for their capacity to inhibit the above enzymes. In our laboratory we initiated screening a variety of structurally diverse small synthetic molecules with the view to identify potent

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inhibitor of Topo II activity. The studies led to the identification of a glycosylated β-amino acid derivative (**I**) as a lead molecule with a weak topo II inhibitory activity. A number of glycoconjugates have been reported in the literature for one or the other activities [11,12]. Compared to standard inhibitors of topo II, sugar derivatives are known to offer better stability, better pharmacokinetic parameters, facilitate the transport of drugs and at the same time are less toxic [13-15]. Thus, the lead molecule (**I**) provides an interesting pharmacophore for the design of more potent inhibitor of topo II. This prompted us to introduce diversity at the amino function of the lead molecule **I** in the first instance and synthesize a library of glycoconjugates with the view to identify congeners with higher inhibitory activity. Libraries of hit molecules both of peptides and of low molecular weight organic molecules have been successfully used in many areas of biomedical research for the purpose of lead optimization [16-20]. For our studies the library represented by general formula X_1-X_2-carbohydrate is designed in a manner wherein various bi-functional amino acids (with no functionality in their side chain) have been introduced at position X_2 whereas at position X_1 both substituted aromatic acids and bi-functional amino acids have been introduced. In the present paper topo II inhibitory activity of *S. cervi* by novel glycoconjugates based on pharmacophore **I** are reported.

EXPERIMENTAL

The libraries were synthesized on Sieber amide resin using a standard method of solid phase peptide synthesis. Fmoc-protected amino acids were purchased from Juro Chemicals (Switzerland). All other reagents and solvents were of standard quality and used without further purification. The pharmacophore **I** was synthesized by 1,4- conjugate addition of ammonia on olefinic ester derived from sugar followed by hydrolysis of amino ester with aqueous ethanolic triethylamine at room temperature. It was used as diastereoisomeric mixture. NMR spectra were recorded on a Bruker 220 MHz instrument. HPLC was carried out using a Shimadzu LC10AS using a C_{18} column (4.6 x 250 mm) with acetonitrile-water as mobile phase.

Fmoc-3-Amino-(3'-O-benzyl-1', 2'-di-O-Isopropylidene-α-D-1', 4'-Pentofuranos-4'-yl)-Propanoic Acid

3-amino-(3'-O-benzyl-1', 2'-di-O-isopropylidene-α-D-1', 4'-pentofuranos-4'-yl)-propanoic acid (2.1 gm, 6.23 mmol) and Na_2CO_3 (0.72 gm, 6.85 mmol) were dissolved in water (10 mL). Fmoc-Osu (2.3 gm, 6.23 mmol) was added to it in dimethoxyethane (35mL), and the mixture was stirred for 16 h at room temperature. Solvent was evaporated to dryness, and the residue was taken up in water (100 mL). The aqueous layer was extracted with ether (2 x 25 mL) and then acidified with $KHSO_4$. A white precipitate was obtained, which was then extracted in ethylacetate (3 x 50 mL) followed by washing with water (2 x 25 mL). The organic layer was dried with sodium sulfate and evaporated to dryness to get Fmoc-3-amino-(3'-O-benzyl-1', 2'-diO-isopropylidene-α-D-1', and 4'-pentofuranos-4'-yl)-propanoic acid as a diastereomeric mixture. Yield 3.44 g (98%), FABMS: 660 $[M+H]^+$. ^{1}H-NMR, 300 MHz($CDCl_3$): δ 1.32, 1.48 ppm (two s, each 3H, $C(CH_3)_2$); 2.66 (m, 2H, CH_2CO_2H); 3.94, 4.05 (two d, 1H, J = 6 Hz, J = 3 Hz, diastereomeric CH(4')); 4.19 (m, 3H, NHCH(3), OCH_2 Fmoc group); 4.43 (d, 1H, J = 3 Hz , CH(3')); 4.47 (d, J = 12 Hz, 1H, $COCH^aPh$); 4.64 (d, J = 3.0 Hz, 1H, CH(2'); 4.69 (d, 2H, J = 12 Hz, $COCH^bPh$); 5.29 (bs, 1H, NH); 5.97 (d,1H, J = 3.0 Hz, CH(1')); 7.34 (m, 9H, Ar-H); 7.59 (m, 3H, Ar-H); 7.76 (m, 2H, Ar-H).

SYNTHESIS OF GLYCOCONJUGATES LIBRARIES ON SIEBER AMIDE RESIN

Material and Methods

Sieber amide resin (0.62 mmol/g loading) was purchased from Juro. For solid phase synthesis, anhydrous DMF and DCM were prepared by the method described in the literature. Loading of Fmoc-3-amino- (3'-O-benzyl-1', 2'-di-O-isopropylidene-α-D-1', 4'-pentofuranos-4'-yl)-

propanoic acid on Sieber amide resin was carried out in a sintered funnel specifically designed for solid phase synthesis. Synthesis of libraries was carried out on Advanced Chemtech 496 MOSΩ in parallel format. For amide bond formation both the TBTu/HOBt and DIC/HOBt methods were used.

Fmoc-3-Amino-(3'-O-benzyl-1', 2'-di-O-Isopropylidene-α-D-1', 4'-Pentofuranos-4'-yl)-propanoyl-Sieber Amide Resin

Sieber amide resin (1.1 g, 0.68 mmol) was placed in a solid phase reaction vessel and treated twice with 20% piperidine-DMF (10 mL each) for 5 min and 25 min at room temperature under N_2 agitation. Next, the resin was successively washed with DMF (3 x 2 min), iPrOH (1 x 2 min) and DMF (3 x 2 min). Fmoc-3-amino- (3'-O-benzyl-1', 2'-di-O-isopropylidene-α-D-1', 4'-pentofuranos-4'-yl)-propanoic acid (1.14 g, 2.05 mmol) was then added to the resin followed by addition of DIPEA (0.67 mL, 4.09 mmol), TBTu (.66 g, 2.05 mmol)) and HOBt (0.31 g, 2.05 mmol) in DMF (10 mL) with N_2 agitation for 3 h at room temperature. Resin was washed with DMF (3 x 2min) and again treated with Fmoc-3-amino- (3'-O-benzyl-1', 2'-diO-isopropylidene-α-D-1', 4'-pentofuranos-4'-yl)-propanoic acid (1.14 g, 2.05 mmol) in the presence of DIC (0.32 mL, 2.04 mmol) and HOBT (0.31 g, 2.05 mmol) for 3 h. The resin was washed with DMF (3 x 2 min), MeOH (3 x 2 min) and DCMF (3 x 2 min). The completion of reaction was monitored by using the Kaiser test.

Synthesis of library

Both Fmoc amino acids and substituted aromatic acids have been used as monomers to build the library in parallel format. The former has been introduced at both the positions X (1) and X (2) of the dipeptide moiety being linked to the carbohydrate unit whereas substituted aromatic acids have been introduced only at position X (1). The following Fmoc amino acids and substituted aromatic acids have been utilized to construct the library.

*Fmoc-Amino acids (Fig **1**)*: Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Cys(Trit)-OH, Fmoc-Nε-Caproic acid, Fmoc-βAla-OH, Fmoc-γAbu-OH, Fmoc-αAbu-OH, Fmoc-Phe-OH, Fmoc-His(Trit)-OH, Fmoc-p-aminobenzoic acid.

*Substituted aromatic acids (Fig **2**)*: 3-Pyridylacetic acid, 3,5 Dimethoxybenzoic acid, Formylbenzoic acid, Cinnamic acid

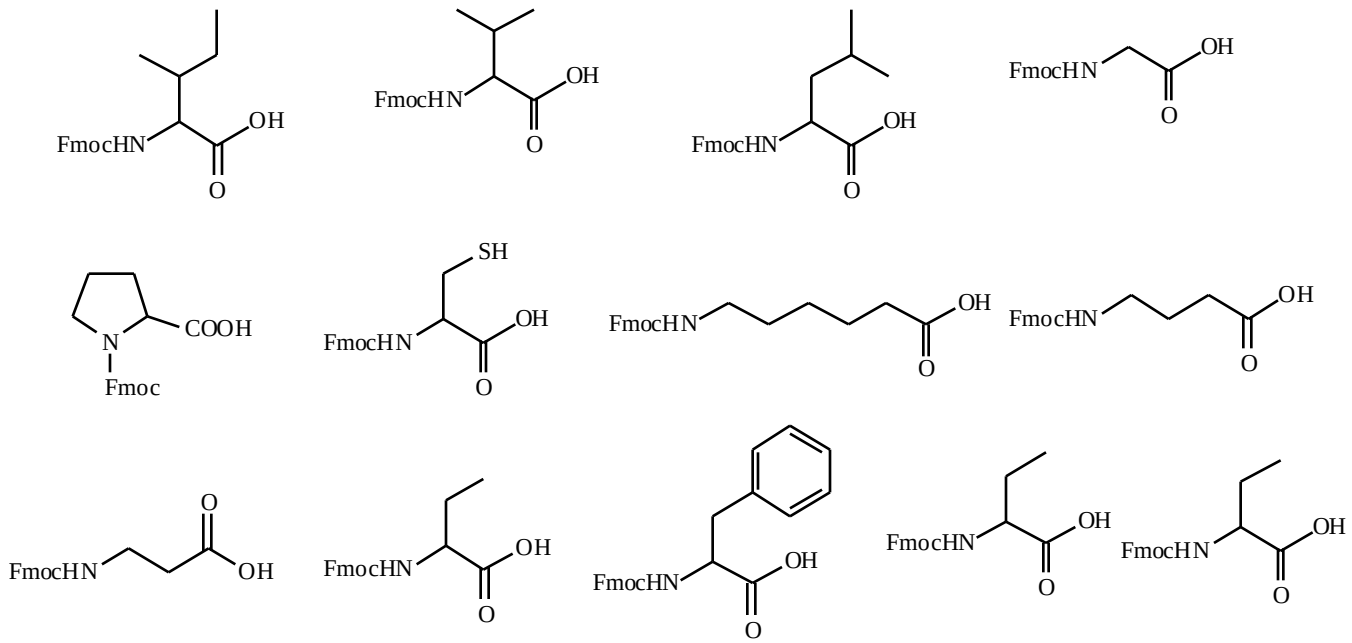


Fig. (**1**). Amino acid building blocks used in the synthesis of glycoconjugates.

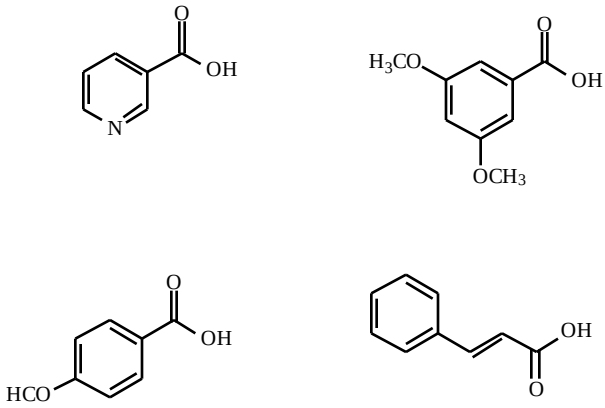
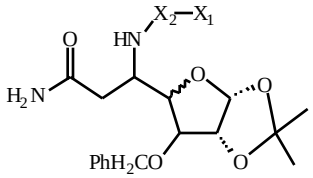


Fig. (2). Substituted aromatic acid building blocks (X_1) used in the synthesis of glycoconjugates.

Procedure for the Synthesis of X_1-X_2-Carbohydrate Moieties

The glycoconjugates (**1-24**; Table I) were synthesized in parallel format in the 96-well reaction block using a 496 MOS Ω automated synthesizer. Fmoc-3-amino- (3'-O-benzyl-1', 2'-diO-isopropylidene-α-D-1', 4'-pentofuranos-4'-yl)-propanoic acyl-Sieber amide resin (0.05 g, 0.031 mmol) was placed in 24 wells in the reaction block and treated with 20% piperidine-DMF (1 mL) twice for 5 min and 25 min. After this, the resin in each well was washed with DMF (3 x 2 min), iPrOH (1 x 2 min) and DMF (3 x 2min). This was followed by transfer of one of the Fmoc amino acids (0.093 mmol) from the list shown above to the desired well and subsequently treatment with DIC (0.10 mL, 0.093 mmol) and HOBt (0.09 g, 0.093 mmol)in DMF for 3 h. After completion, the resins were again washed using the same wash chemfile as described earlier. The resin in each well was then treated for 3 h with the desired Fmoc amino acid using the DIC/HOBt method. Completion of reaction was determined by a negative ninhydrin test. This was followed by removal of Fmoc group using 20% piperidine-DMF. Next, treatment the with desired substituted aromatic acids or Fmoc amino acid was carried out to complete the synthesis. The reaction was again monitored by using theninhydrin test.

Table 1. Structure of Variants X_1-X_2 Introduced at the Amino Function of the Carbohydrate Moiety I



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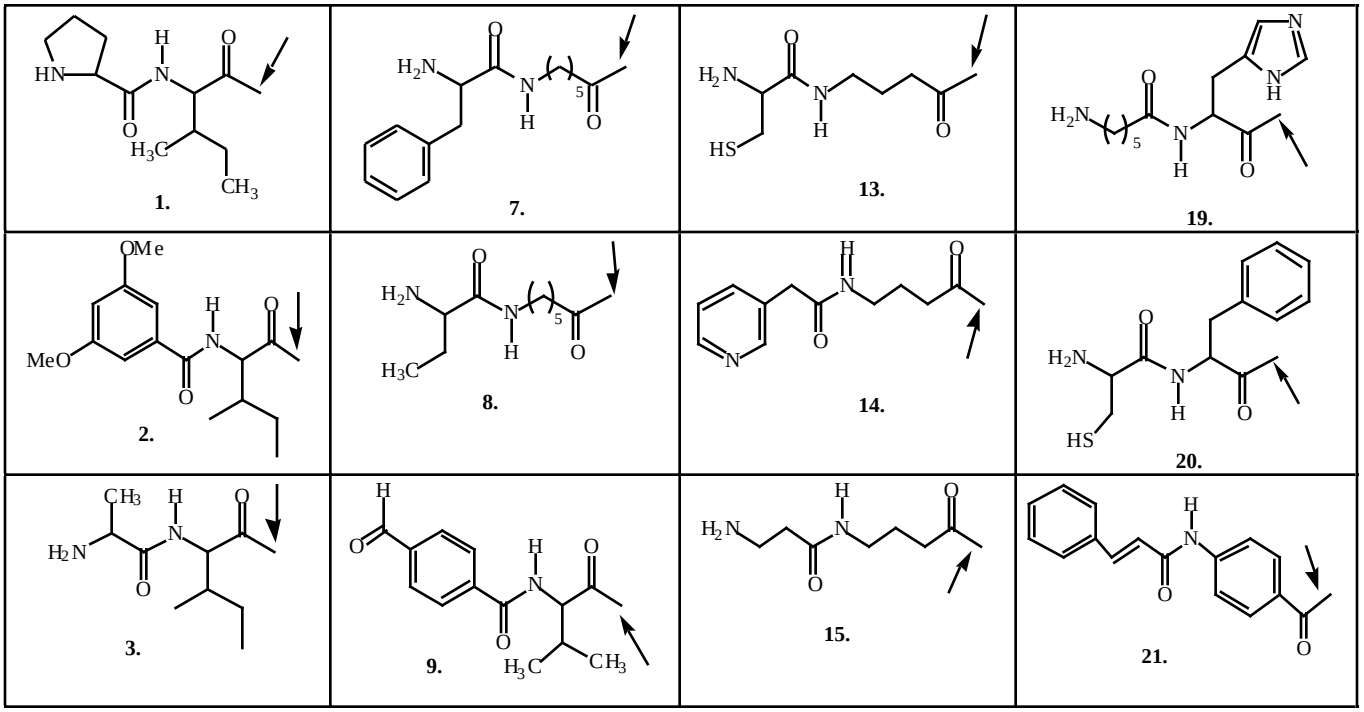
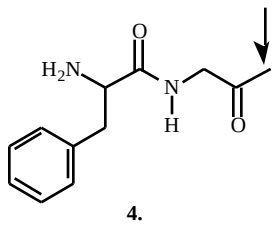
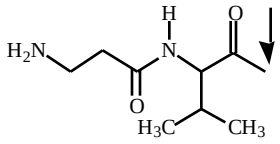
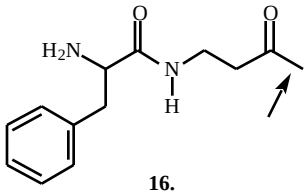
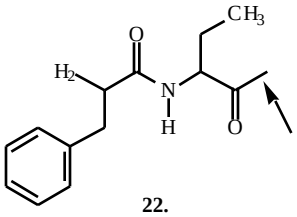
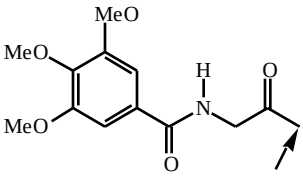
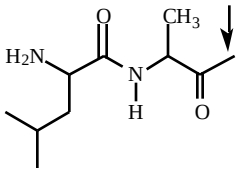
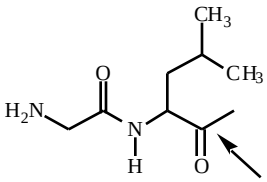
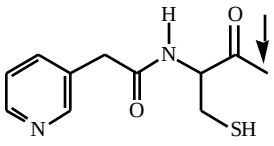
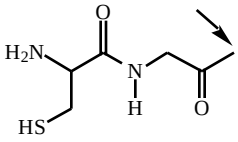
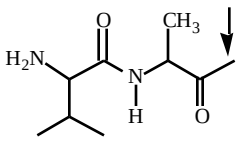
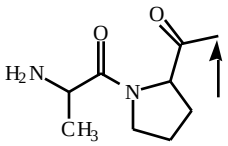
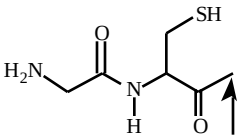


(Table 1). contd.....

 **4.**	 **10.**	 **16.**	 **22.**
 **5.**	 **11.**	 **17.**	 **23.**
 **6.**	 **12.**	 **18.**	 **24.**

Indicates point of attachment to the carbohydrate moiety **I**

The resins in wells **1, 3, 4, 6, 7, 8, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 22 and 24** were treated with 20% piperidine-DMF to remove the Fmoc group followed by washing as described above. Finally the glycoconjugates were cleaved from the resin using mixture of 2% TFA, 94% dichloromethane and 4%triisopropylsilane. After deprotection and cleavage, the final compounds were lyophilized by dissolving them in t-butanol/ water. The compounds were characterized using HPLC, FAB mass spectrometry and proton NMR. The glycoconjugates (**1-24**) were obtained in 85-90% purity, which was sufficient for *in vitro* evaluation.

DNA topoisomerase II estimation

The reaction catalyzed by DNA topoisomerases II was measured as reported previously [7]. The standard topoisomerase II reaction mixture was contained in a final volume of 20 µL and consisted of 50 mmol Tris-HCl (pH 7.5), 50 mmol KCl, 10 mmol $MgCl_2$, 1 mmol ATP, 0.1 mmol EDTA, 0.5 mmol DDT, 30 µg /mL BSA, 0.25 µg pBR322 and enzyme protein. The reaction was stopped by adding 5 µL of stock buffer followed by electrophoresis of samples on 1% agarose-gel in Tris-acetate buffer for 18 h at 20 V. Gels were stained with ethidium bromide (0.5 µg/ mL) and visualized and photographed on a GDS 7500 UVP (Ultra Violet Products, UK) transilluminator. The effect of inhibitors on the enzyme activity was measured by incubating enzyme protein with inhibitor (40 µg/reaction mixture) for 10 min at 37°C and starting the reaction by addition of pBR322. The percent inhibition was measured by measuring the microdensitometry of the gel with a Gel Base/Gel Blot Pro Gel analysis software program. The results obtained for various glycoconjugates are summarized in Table II and compared with the lead compound **I**, novobiocin and nalidixic acid.

RESULTS AND DISCUSSION

Out of the 24 glycoconjugates screened for their ability to inhibit DNA topoisomerase II, nine congeners exhibited low to marked order of inhibitory activity (Figs. **3** and **4**). At the N-

Table II. Effect of Glycoconjugates on DNA Topoisomerase II of Filarial Parasite *Setaria cerv*

Compound No.	Percent inhibition
Enzyme alone	nil
Enzyme + **1**	nil
Enzyme + **2**	35
Enzyme + **3**	nil
Enzyme + **4**	nil
Enzyme + **5**	nil
Enzyme + **6**	95
Enzyme + **7**	2
Enzyme + **8**	nil
Enzyme + **9**	nil
Enzyme + **10**	nil
Enzyme + **11**	nil
Enzyme + **12**	nil
Enzyme + **13**	50
Enzyme + **14**	75
Enzyme + **15**	n.d.
Enzyme + **16**	11
Enzyme + **17**	15
Enzyme + **18**	15
Enzyme + **19**	22
Enzyme + **20**	23*
Enzyme + **21**	n.d.
Enzyme + **22**	20
Enzyme + **23**	26*
Enzyme + **24**	23*
Enzyme + Lead compound **I**	30
Enzyme + Novobiocin (at 100nmols)	100
Enzyme + Nalidixic acid (at 100nmols)	90
Enzyme + DEC (at 100nmols)	80
Enzyme + Ivermectin (at 100nmols)	40

*The compounds formed complex with DNA; n.d. = not determined; Compounds 1-24 were used at 40 μg/reaction mixture.

terminal region of the glycoconjugates, Cys appears to be the most preferred moiety responsible for the inhibitory activity. It is interesting to note that none of the congeners with Cys next to the carbohydrate moiety exhibited any activity.

Compounds **13** and **14** exhibited moderate inhibitory activity of 75% and 50% respectively (Fig. **3**, lane 15 and 16), and they were more active than the lead compound **I** which exhibited only 30% inhibition of DNA topo II. It is interesting to note that replacement of Cys in compound **13** by a

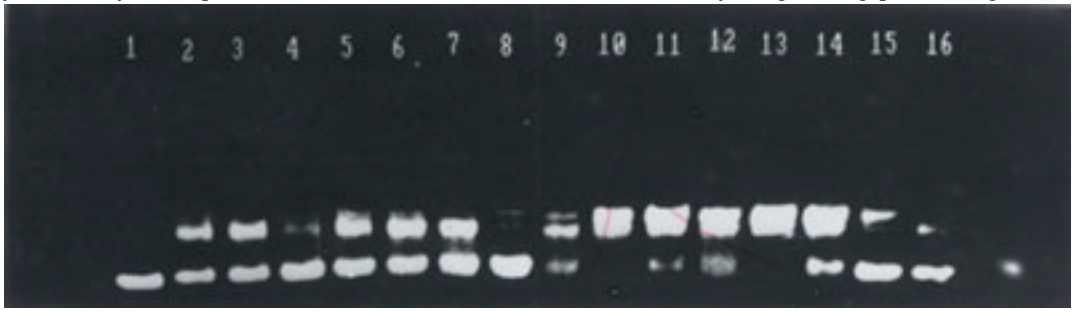


Fig. (3). Inhibition of *S. Cervi* topoisomerase II by glycoconjugates. The enzyme activity was monitored as described in the text. Lane 1 pBR322 DNA alone; Lane 2 DNA + *S. Cervi* topoisomerase II; Lane 3-16 identical to Lane 2 with glycoconjugates **1**-**14**.

pyridylacetyl moiety resulted in compound **14** which exhibited a drop in the inhibition from 75% to 50%. This suggests that Cys at the N-terminal region plays a crucial role in the inactivation of the enzyme. The most active compound of the series was compound **6** with Cys-Gly adornment, which exhibited 95% inhibition of DNA topo II (Fig. **3**, lane **8**). Thus, the introduction of a Cys-Gly moiety at the amino function in **I** led to at least a 3-fold increase in topo II inhibitory activity compared to lead molecule **I**.

Compounds 20, 23 and 24 formed complexes with the DNA resulting in enzyme inhibition (Fig. **4**, lane 7, 9 and 10). This inhibition might be attributed to the low availability of the binding sites for the enzyme on the DNA. Compound 6 was further tested at different concentrations, and it exhibited 90-100% inhibition at 1.09 μM whereas 50% inhibition was observed at 0.54 μM. In contrast, nalidixic acid and novobiocin exhibited 90% and 100% inhibition at 5.0 μM. Similarly, the established antifilarials DEC and ivermectin showed 80 and 40% inhibition respectively at 5.0 μM. Thus, compound 6 appears to be at least five times more potent than the known inhibitors nalidixic acid and novobiocin.

CONCLUSION

Our studies using a combinatorial approach thus provide a novel low molecular weight pharmacophore with potent DNA topo II inhibitory activity. Since no satisfactory inhibitor for topo II of*S. cervi* is currently available, glycoconjugate **6** lays foundation for the design of more potent inhibitors.

ACKNOWLEDGEMENTS

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Fig. (4). Inhibition of*S. cervi* topoisomerase II by glycoconjugates. The enzyme activity was monitered as described in the text. Lane 1, pBR322 DNA alone; Lane 2, DNA + *S. Cervi* topoisomerase; Lane 3 – 7, identical to Lane 2 with glycoconjugates **16**-**20**; Lane 8 –10, identical to Lane 2 with glycoconjugates **22**-**24**.

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