Novel purine thioglycoside analogs: synthesis, nanoformulation and biological evaluation in in vitro human liver and breast cancer models

Background: A series of novel pyrazolopyrimidine and pyrazolopyridine thioglycosides were synthesized and confirmed via their spectral analyses.

Purpose: To evaluate the effect of these anti-metabolic compounds against proliferation of Huh-7 and Mcf-7 as in vitro models of human liver and breast cancers, respectively. Vero cells were used as an example of normal green monkey kidney cells.

Methods: The most promising compound was subjected to a nanoformulation by its encapsulation into chitosan nanoparticles to increase its anti-cancerous activity. Nanoformulation was confirmed by TEM and FT-IR to ensure encapsulation and screened for their cytotoxicity against Huh-7 and Mcf-7 cells using MTT colorimetric assay and morphological examination. Genotoxic effect was performed by cellular DNA fragmentation assay. Simulated CompuSyn software (linear interaction effect) was conducted to predict the possible synergistic effect of nanocomposite as anticancerous activity. Apoptotic effect was further analyzed by detection of apoptotic proteins using ELISA assay.

Results: The nano preparation was successfully prepared by encapsulation of compound 14 into chitosan nanoparticles, controlled to a size at 105 nm and zeta charges at 40.2 mV. Treatment of Huh-7 and Mcf-7 showed that compound 14 was the most cytotoxic compound on both cancer cell lines where IC$_{50}$ was 24.59 (9.836 μg/mL) and 12.203 (4.8812 μg/mL) on Huh-7 and Mcf-7, respectively. But IC$_{50}$ of the nano preparation was 37.19 and 30.68 μg/mL on Huh-7 and Mcf-7, respectively, indicating its aggressiveness on human breast cancer cells as confirmed by DNA fragmentation assay and theoretically by CompuSyn tool.

Conclusion: A novel series of pyrazolopyrimidine thioglycosides and pyrazolopyridine thioglycosides were synthesized. Nanoformulation of compound 14 into chitosan nanoparticles demonstrated anticancer activity and can be used as a drug delivery system, but further studies are still required.

Keywords: purine thioglycoside analogs, chitosan nanoparticles, human breast cancer cells, human liver cancer cells, anticancer agents, antimetabolites

Introduction

Interest in the synthesis of antipyrine derivatives has been intensified following the discovery of their various biological applications. They have been reported to have diverse pharmacological activities, such as antimicrobial, antiviral, anticancer, antioxidant, antipyretic, analgesic and anti-inflammatory activities. Cancer disease has been turned out to be one of the world’s primary general medical issues. Early attack, invulnerable escape, early metastasis and other natural exhibitions are the inconvenience of tumor treatment. At the point when the tumor has been found in the dynamic stage, the tumor has the ability to be exchanged to a phase in which it became so difficult to be
eradicat ed by medical procedure and simple to relapse after medical procedure. The old treatment methods are surgery, radiation, and chemical treatment, which is associated with high-risk destruction to neighboring tissues, infection, pain and relapse of cancer. A less aggressive choice is radiation therapy, showed side effects such as skin changes, faecal incontinence, diarrhea, nausea and vomiting which obviously affects public health. Further down the line in terms of non-invasiveness is chemotherapy, which properly is the first choice in the administration of cancer. However, chemotherapy is also connected with side effects such as pain sores in the mouth and throat, nausea and vomiting as well as blood disorders. In order to relieve some of the side effects due to chemotherapy, there was a growing interest in the use of nanotechnology as an alternative approach for cancer treatment. The purpose of overcoming the adverse effects of drugs is by encapsulating the chemotherapeutic drug into the nanodelivery system. Advancement has been made in the treatment of cancer by conjugating chemotherapeutic agents into nanocarriers. These nanocarriers are often made from polymeric materials such as poly(lactide-co-glycolide) (PLGA), chitosan and poly-hydroxyethyl methacrylate/ stearic acid. Chitosan (Cs) is the essential polysaccharide elucidated by deacetylation of chitin, which must be the most copious common biopolymer on the earth with the exception of cellulose. Chitosan has been broadly utilized in the pharmaceutical production due to its low lethality, biodegradability, and biocompatibility. In recent years, Chitosan was significantly used for the delivery of active pharmaceutical compounds. It tends to be utilized to shape nanocarriers for drug delivery, in particular feebly dissolvable therapies or biotechnology-based medications. The two frameworks can protect the medication from being broken down inside the body. Polymeric conveyance frameworks can change the pharmacokinetics of medication, prompting a propelled helpful list by diminishing the symptoms and developing viability. Small size and large surface area to volume ratio of the chitosan nanoparticles gave it its unique characteristics. Chitosan nanoparticles showed to be promising carriers for several drugs specially hydrophobic drugs in cancer drug delivery application. Chitosan and sodium alginate were utilized to uploading 5-FU (anti-metabolic medication) by ion gelation method and had maintained discharge of 5-FU in a controlled way. Karolyn Infanta and David have performed their in vitro study by successful encapsulating quercetin and 5-FU into chitosan nanoparticles and hence its sustainable release. Such strategies created an impressive consideration because of its associated efficacy, improving drug properties, helpful and controllable. Ionic gelation procedure depends on the ionic communications between the decidedly charged essential amino gatherings of chitosan and the contrarily charged gatherings of polyanion, for example, sodium tripolyphosphate (TPP), which is the most broadly utilized particle cross-connecting operator because of its non-dangerous and multivalent properties. This physical cross-connecting strategy not just keeps away from the utilization of substance cross-connecting and emulsifying agents which are regularly dangerous to the organ, in addition stays away from the likelihood of drugs impairment. In the light of the abovementioned results along with our previous reports for synthesizing novel antimetabolic agents and heterocyclic thioglycosides, the purpose of this work is to design, synthesize and investigate the anti-tumor activity of antipyrine derivative and fused antipyrine bearing carbohydrate moieties by forming S-glycosidic bonds. To our knowledge, this is the first method in which the preparation of such a novel class of 4-aminooantipyrines will be reported. Also, in this study, we choose to use LMW chitosan with high degree of deacetylation, and concentration on their producible construction of (Chitosan–TPP) and (Chitosan-14- TPP) nanoparticles, which desired to stimulate the development of (Chitosan–TPP) nanoparticles in the applications of drug delivery for our synthetic compounds.

Materials and methods
Low molecular weight chitosan (Cs, Deacetylation degree of ≥75%, viscosity 20300 – cps and molecular weight of 50190 – kDa), sodium tripolyphosphate (TPP) were purchased from Sigma Aldrich (St. Louis, MO, USA), acetic acid glacial.

Preparation of (Cs–14) nanoparticles
Chitosan nanoparticles were prepared according to a modified method of Calvo et al based on the ionic gelation of chitosan with TPP anions. Briefly, a specific amount of Cs (1 mg/mL) was dissolved in 1% acetic acid solution and TPP solution (10 mg/mL). Nanoparticles were obtained by mixing Cs solution with TPP for a volume ratio of [3:1], respectively. Compound 14 powder which was accurately weighed dissolved in DMSO to prepare a concentration of (1 mg/mL) solution. A 300 µL aliquot solution from the prepared compound 14 solution was then slowly dropped into the Cs solution using a micro-syringe under mechanical stirring for (600 rpm) for (30 mins). Finally, sodium tripolyphosphate solution was added to the above mixture under mechanical stirring (600 rpm) for (1 hr). The reaction was kept for 1.5 hrs at room temperature. Chitosan nanoparticles were prepared according to a modified method of Calvo et al based on the ionic gelation of chitosan with TPP anions. 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nanoparticles (Cs NPs) without compound 14 were prepared by the same method.

Nanoparticles characterization
Size and zeta potential measurement
Particle size and zeta potential, Z-average diameter of the prepared nanoparticles and their zeta potential were determined by the Malvern particle size analyzer (Model-Nano ZS, Malvern Instruments limited, UK). Triplicate samples were analyzed and the mean value was reported.

Measurement of particle size and morphology by TEM
Nanoparticles morphology was examined by TEM apparatus (JOEL-JEM–1010, Japan) at an accelerating voltage of 100 kV. Nanoparticles suspension was properly diluted. A drop was withdrawn with a micropipette then placed on a carbon-coated copper grid. The excess of the suspension was removed by blotting the grid with a filter paper. Then, the deposit was left to dry before analysis.

Fourier transform infrared spectroscopy (FTIR)
In order to confirm the potential chemical interaction happening between (Cs–TPP) NPs and (Cs–compound 14–TPP) NPs, FTIR spectra of compound 14, (Cs–TPP) nanoparticles and compound 14 loaded Cs nanoparticles were taken using (Perkin Elmer, USA) Fourier transform infrared spectrophotometer.

Cell culture
Established human liver cancer cell line (Huh-7) and human hormonal breast cancer cell line (Mcf-7) were acquired from American Tissue Culture Collection, USA. The cells were kept up in DMEM media (Lonza) supplemented with heat inactivated 10% fetal bovine serum (Gibco), and anti-microbials (2% penicillin-streptomycin [Gibco] and 0.5% fungizone [seralab]). The cells were kept up in monolayer culture at 37 °C under a humidified air of 5% CO2. The cells were passaged by trypsinization (0.025% trypsin and 0.0025% EDTA; Biowest), and kept up in tissue culture laboratory of Virology & Immunology Unit, Cancer Biology Dept. at the National Cancer Institute, Cairo University, Egypt. Cell numbers and viability were observed by standard Trypan blue color (Gibco).

Cytotoxicity assay (MTT)
The anti-cancerous activity of the synthesized compounds was determined against a human liver cancer cell line (Huh-7) and breast cancer (Mcf-7) using Doxorubicin as a reference drug and (Serva electrophoresis, Germany) MTT assay based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark violet insoluble formazan crystals. Data generated were used to plot a dose–response curve from which the concentration of test compounds required to kill 50% of cell population (IC50) was determined. Cells were seeded in 96-well plate at a cell concentration (6×10³) cells per well in 100 μL of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 hrs of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed in 96-well, flat-bottomed microtiter plates using a multichannel pipette. Control cells were incubated without test sample with DMSO (0.1% v/v). After incubation of the cells for 24 hrs at 37°C, various concentrations of sample (100, 50, 25, 12.5 and 6.25 μM/mL) were added, the incubation was continued for 48 hrs. At the end of experiment, MTT solution in PBS (5 mg/mL) was then added to all wells, and left to incubate for 2 hrs. The formation of formazan crystals were visually confirmed using phase contract microscopy. DMSO (100 μL/well) was added to dissolve the formazan crystals with shaking for 10 mins, after which the absorbance was read at 570 nm on ELISA microplate reader (Tecan Sunrise ELISA Reade, USA). Cell proliferation was calculated comparing the OD values of the DMSO control wells and those of the samples both represented as % viability to the control.

DNA fragmentation
Fragmentation of cellular DNA was investigated following treatment of Mcf-7 and Huh-7 cell lines with compounds 3, 4, 8a, 6b, 8b, 9b, 13, 15, 14 and 16 at high concentrations (100 μM) and (Cs–14) NPs at concentration 100 μg. A fixed amount (350 ng) of cellular DNA (GeneJET Genomic DNA purification kit, Thermo Scientific) extracted from treated and untreated cells was subjected to 1.5% agarose gel electrophoresis in TEA buffer, stained with 5 μL ethidium bromide. The bands were examined under UV transillumination and photographed. Smearing, or presence of many low molecular weight DNA fragments, is a characteristic feature of apoptotic cells.
Drug combination analysis using median effect principle

The nature of interaction of both compounds (Cs NPs), compound 14 in vitro was determined by treatment of (Mcf-7 and Huh-7) cells with specific concentration. The cells were seeded at a density of 6,000 cells/well and allowed to adhere overnight. All compounds were administered at a concentration (100 µg) and incubated for 48 hrs in a humidified chamber at 37°C. After incubation, MTT assay was performed as described previously. All possible combinations of the two compounds within this concentration were analyzed for any additive, synergistic or antagonistic effects. The data were analyzed for combination index using CompuSyn software.

Detection of apoptotic caspase-3 and anti-apoptotic Bcl-2 proteins levels

Caspase-3 activity was measured after treatment of cells (Mcf-7 and Huh-7) with compound 14 at concentration of 100 µg. Caspase-3 activity was measured using colorimetric Bender Med System (Caspase 3 assay kit; BMS2012INST) while Bcl-2 activity was measured using colorimetric abcam (Human Bcl-2 ELISA Kit(ab119506). Six well plates were seeded with (3×10^4) cells and incubated overnight under optimum culture conditions before treatments with the estimated (IC_{50}) of the selected compounds in relation to doxorubicin as standard chemotherapeutic agents. The cells were harvested and total proteins were isolated. Protein levels of the apoptotic (Caspase-3) and anti-apoptotic marker (Bcl-2) were then measured using ELISA according to the manufacturers’ instructions (eBioscience, USA). Standard curves were drawn for each kit. The reaction products were measured at 450 nm using ELISA reader (Tecan sunrise microplate reader, Awareness Technology Inc, Minnesota, USA).

Results and discussion

Chemistry

The synthesis was started by the reaction of 4-aminoantipyrine 1 and sodium cyanocarbonimidodithioate salt 2 in acetic acid (Scheme 1). The reaction mixture was refluxed for 5 mins producing compound 3 in a quantitative yield. The structure of the synthesized compound 3 was elucidated on the basis of its spectroscopic data. The latter reacts with α-acetobromoxyllose 5a and α-acetobromoarabinoside 5b in acetone-KOH at room temperature to give the corresponding S-xyloside 6a or S-arabinoside 6b (Scheme 1). The structures of reaction products 6a,b were confirmed by their spectroscopic data and basic analysis (13C NMR, 1H NMR and IR). The 1H NMR spectrum revealed the anomeric proton of 6a as a doublet at δ 5.42 ppm. The coupling constant (J_{1,2}=8.2 Hz) indicated H-1' to be trans-diaxial to H-2', and the other five xylose protons resonated at 3.56–5.13 ppm and showed three acetyl groups as three singlets at δ 1.98–2.06 ppm. The 13C NMR spectrum of 6a contained a signal at δ 78.54 corresponding to the -C-1' atom and four signals appearing at δ 60.36, 68.61, 75.47 and 76.73 which were set to -C-5', -4', -C-3' and -C-2', respectively. When glycosides 6a, b were reacted with NH_2-MeOH at 0–5°C for 10 mins provided the free hydroxyl derivatives 8a, b (Scheme 1), the structures of which were confirmed based on their spectroscopic data. Thus, 1H NMR spectra of 8a shows the anomeric proton as doublet at δ 5.34 (J_{1,2}=10.22 Hz), indicating only the β D-configuration and three hydroxy groups of xylose resonance at δ 4.99–5.12 (exchangeable by D_2O). Cyclization of compound 3 in the boiling of EtOH/HCl resulted in the formation of the 7-imino-5-mercaptop-pyrazolo [4, 3-d] pyrimidin-3(2H)-one 4 (by mass spectra). The 1H NMR spectrum revealed an imine group (D_2O exchangeable) and two different methyl protons, demonstrating that the methyl group at C-5 did not participate in the cyclization. A similar intramolecular cyclization reaction of this type was reported by us. In another experiment, compound 4 reacted with α-acetobromoxyllose 5a and α-acetobromoarabinoside 5b in acetone at room temperature to give the corresponding S-xyloside 7a or S-arabinoside 7b, respectively. It was suggested that cis (α) sugar be reacted by a simple SN2 reaction to give a β glycoside product. The structures of the 7a,b reaction products were confirmed by elemental analyses and spectral data (13C NMR, 1H NMR, IR). Thus, the 1H NMR spectrum for 7a showed the anomeric proton as a doublet at δ 5.56 ppm with a spin–spin coupling constant of 10.6 Hz which corresponds to the dixial orientation of H-1' and H-2' protons indicating the β configuration. The formation of 1,2-trans glycosides is strongly favored by the neighboring group involved. Typically, the use of a co-replacement substituent at C-2 is a support to justify stereoselective 1,2-trans glycosylation. The 13C NMR spectrum of 7a contained a signal at δ=79.58 corresponding to the -C-1' atom of the β-configuration. Four signals appearing at δ=62.11, 69.27, 71.86 and
Scheme 1 A synthetic pathway for pyrazolopyridine thioglycosides 7a,b and 9a,b.
73.75 were set to C-5', C-4', C-3' and C-2' respectively. After the removal of protecting groups of compounds 6a, b and 7a,b with a NH₃/MeOH solution at room temperature, the final free glycosides 8a,b and 9a,b were obtained and their structures were confirmed on the basis of their spectroscopic data. Thus, the ¹H NMR spectrum of 9a shows the anomeric proton as a doublet at δ 4.66 (J₁-2=10.2 Hz) indicating that only the β D-configuration exists and the other five protons of xylose appear at δ 3.78–4.41, while the three hydroxyl xylose signals are observed at δ 4.91–5.23 (exchangeable by D₂O). The ¹³C NMR spectrum of 9a showed a signal at δ 81.93 corresponding to the -C-1' atom of β -D-xylopyranose, and four other signals at δ 62.41, 69.28, 71.62 and 74.92 are assigned to C-5', C-4', C-3' and C-2' respectively. In order to investigate the scope of this reaction, and in order to determine whether the reaction of sodium salts of cyanocarbonimidodithioate or cyanoketene dithioacetals with 4-amino-pyrazol-3(2H)-5-one could be extended to provide a general approach to fused pyrazole thioglycosides, we studied the reaction of 4-aminoantipyrine 1 with functioned ketene dithioacetals (Scheme 2). Thus, compound 1 reacts with sodium 2,2-dicyanoethene-1,1-bis(thiolate) 10 in refluxing acetic acid for 5 mins to yield the acyclic structures 11. The latter undergoes intramolecular cyclization on refluxing in EtOH/HCl to afford the corresponding 5- mercapto-pyrazolo[4,3-b]pyridines 12. The structure of 12 was confirmed on the basis of its spectroscopic data (¹³C NMR, ¹H NMR, and IR). The ¹³C NMR spectrum of 12 was characterized by two methyl carbons at 12.13 (CH₃) and 40.42 (N-CH₃) ppm. Compounds 11 and 12 bear a mercapto group which may be useful for the synthesis of 4-aminoantipyrine thioglycoside and their corresponding pyrazolo[4,3-b]pyrimidine-5-thioglycosides. Thus, it has been found that the reaction of the structures 11 and 12 with α-acetobromoxylene 5a and α-acetobromoxylarabinose 5b in aceton/ KOH at room temperature gives the corresponding thioglycosides 13 and 14. The structures of 13 and 14 were confirmed based on spectral data (¹³C NMR, ¹H NMR, IR). Thus, the ¹H NMR spectrum for compound 14 showed the ring imine proton at δ 5.66 ppm and the anomeric xylose proton as a doublet at δ 5.53 ppm with a spin–spin coupling constant of 10.4 Hz which corresponds to the diaxial orientation of H-1' and H-2' protons indicating the β configuration. The formation of 1,2-trans glycosides is strongly favored by the neighboring group involved. Typically, the use of a co-replacement substituent at C-2 is a support to justify stereoselective 1,2-trans glycosylation. The ¹³C NMR spectrum of 14 contained a signal at δ=78.81 corresponding to the -C-1’ atom of the β configuration. Four signals appearing at δ=62.63, 68.74, 72.64 and 74.22 were assigned to C-5’, C-4’, C-3’ and C-2’ respectively. The IR spectrum of compound 14 showed the three ester CO groups at ν 1751 cm⁻¹ and the ring CO at ν 1630 cm⁻¹. The deprotection of the protected thioglycosides 13 and 14 with a solution of NH₃/MeOH at room temperature lead to the formation of the final free thioglycosides 15, 16. The structures of compounds 15, 16 were proved according to their spectral data and chemical analysis. Thus, the ¹H NMR spectrum for the free pyrazolopyrimidine thioglycoside 16 revealed the anomeric xylose proton as a doublet at δ 5.24 (J₁-2=10.23 Hz), indicating the existence of the β-D-configuration. The other five xylose protons appear as a multiplet at δ 3.63–4.55, while the three hydroxyl groups of xylose moiety resonated at δ 4.91–5.01 (exchangeable by D₂O). Antitumor activity of the synthesized compounds against human tumor cell lines has been tested; liver (Huh-7) and breast (Mcf-7).

Fourier transform infrared spectroscopy (FTIR) characterization

The FTIR spectra of the synthetic compound 14 and formulated nanoparticles are presented in Figure 1. In the spectra (a), stretching vibrations of (C=O), and (≡N–H) of compound 14 appears at 1,751 and 3,438 cm⁻¹, respectively. For chitosan nanoparticles, the peak of amide I (NH₂ bending) appeared at 1,643 cm⁻¹, d peaks appeared at 1,226 (C–O–C stretch) and 1,543 cm⁻¹ (amide II), implying the complex formation via electrostatic interaction between NH₃⁺ groups of chitosan and phosphoric groups of TPP within the nanoparticles. The characteristic peak at 1,086 cm⁻¹ is assigned to (P=O) groups of TPP, while the one at 897 cm⁻¹ is related to the (P-O-P) asymmetric stretching. These bands were all present in both the formulations (Cs NPs) and (Cs–14) NPs, spectra (b and c, respectively). We may conclude that these groups are not typically involved in covalent chemical bonding with the other components during the formulation process. The (Cs–14) NPs FTIR spectrum is slightly similar to the (Cs NPs) spectrum except for a slight shifting of the amine peak at 1,633 cm⁻¹ which is attributed to the loading of compound 14 on (Cs NPs). Furthermore, the peak attributed to compound 14 is absent in the (Cs–14) NPs spectrum, which assures loading compound 14 in the latter.
Scheme 2 A synthetic pathway for pyrazolopyrimidine thioglycosides 14 and 16.
Determination of particle size by zetasizer and stability studies by zeta potential measurements

The particle size distribution, PDI and zeta potential of (Cs NPs) were measured by Zeta-Sizer instrument, which shows a size 44 nm, 0.457 and 38.5 mV, respectively while those for (Cs–14) NPs were 105 nm, 0.418 and 40.2 mV. The particle size distribution via Zeta-Sizer for the compound 14 loaded (Cs NPs) nanoparticle is represented in Figure 2. These zeta potential values confirm the good stability of both (Cs NPs) and compound 14 loaded (Cs NPs) nanoparticle and the positive zeta potential reveals the positive surface charge in both of the systems. The zeta potential of (Cs NPs) nanoparticles was increased with compound 14 loading; this can be elucidated by the effective interaction chemistry between compound 14 and the (Cs NPs) nanoparticles. Once loaded within the (Cs NPs) nanoparticles, compound 14 enhanced an additional positive charge to the loaded (Cs NPs) nanoparticles as it can have a net positive charge (outcomes from the protonation of the (=N–H)). Function group in the pyrimidine ring within compound 14 as well, this added up the surface charge. The interesting result here is that, the zeta potential itself is a good evidence for the conceivable interaction chemistry of compound 14 with (Cs NPs) nanoparticles.

Morphology of (Cs NPs) and (Cs–14) nanoparticles

The morphology of the particles was detected by transmission electron microscopy (TEM). The TEM images of the (Cs NPs) and (Cs–14) NPs exhibited regular distribution and spherical shape that show and have a particle size distribution in the range (20–35) and (40–70) nm, respectively, as shown in Figure 3. This divergence in the size of (Cs NPs) and (Cs–14) NPs between Zeta-sizer and TEM may be according to that (Cs NPs) and (Cs–14) NPs expand in aqueous media and Zeta-sizer provides a hydrodynamic diameter of nanoparticles, whilst TEM gives a definite diameter of NPs in dry state. Whilst the aggregation of the (Cs NPs) and (Cs–14) NPs is possible because that the hydrogen bonding contacts amongst (Cs NPs) gradually become dominant in the drying process.52
Biological activity

Evaluation of the cytotoxic effect of the 16 novel synthesized compounds comparing to doxorubicin (as a reference antitumor drug) was performed in vitro for their anticancer activities against human cancer cells according to the standard procedures as described in the experimental part. The tumor cell lines have been used included; human hepatocellular carcinoma (Huh-7), breast cancer cell line (Mcf-7) and green monkey kidney cell lines (VERO) as examples of the normal cells. Our result showed that compound 14 was the most cytotoxic compound that could inhibit proliferation of human liver cancer cells Huh-7 where IC\textsubscript{50} was found to be 24.59 \(\mu\text{M}\), followed by compounds 16 and 15 where their IC\textsubscript{50} values were 26.64 and 30.67 \(\mu\text{M}\), respectively.

Whereas the following compounds; 6a, 6b, 8b, 7a and 13 displayed moderate and mild cytotoxic effect on Huh-7 giving IC\textsubscript{50} values of 89.43, 64.52, 83, 92.42 and 42.9 \(\mu\text{M}\), respectively. But upon testing those compounds on human breast cancer cells (Mcf-7), our results revealed that compounds 14, 16 and 13 were the most cytotoxic compounds against Mcf-7 with IC\textsubscript{50} values of 12.203, 14.84 and 26.66 \(\mu\text{M}\), respectively. Whereas compound 15 showed moderate cytotoxic effect with IC\textsubscript{50} of 55.026 \(\mu\text{M}\). But compound 8b showed a mild cytotoxic effect with IC\textsubscript{50} of 71.38 \(\mu\text{M}\).

Regarding the remaining compounds, they revealed no marked cytotoxic effect IC\textsubscript{50} > 100 \(\mu\text{M}\) (Table 1). While the cytotoxicity effect of (Cs–14) NPs showed us moderate effect on both cell lines (Huh-7 and Mcf-7) with IC\textsubscript{50} of 37.19 \(\mu\text{g}\), 30.68 \(\mu\text{g}\) for (Cs–14) NPs, respectively, as shown in Table 2. Notably, the structure–activity relationship of the 16 newly synthesized compounds revealed that the cyclized thioglycosides compounds 14 and 16 showed higher anti-proliferative activity against both Huh-7 and Mcf-7 cell lines than the acyclic thioglycosides 13 and 15. In addition, pyrazolopyrimidine thioglycosides 14 and 16 had higher antiproliferative activity than pyrazolopyridine thioglycosides 7a, 9a, 7b and

**Figure 2** The particle size distribution ion and zeta potential of (A) chitosan NPs and (B) (Cs–14) NPs.

**Figure 3** TEM image of (Cs NPs) (A), (Cs–14) NPs (B).
The combination index indicates whether the two compounds interact in an additive (CI=1), synergistic (CI<1) or antagonistic (CI>1) manner.55 Tables 3 and 4 and Figures 6 and 7 elaborate the CI grades appointed for different combinations of (Cs NPs) and compound 14 on both cell lines (Huh-7 and Mcf-7). The arranging for the CI values has been achieved as recommended by Chou.56 The median effect analysis and calculation of combination index of free (Cs NPs) and compound 14 using the CompuSyn software showed that (Cs NPs) and compound 14 produced different results with different cell lines.

**Cellular DNA fragmentation assay**

Further investigation on a molecular level was applied by DNA fragmentation assay to investigate genotoxic effect which in turn demonstrates the late apoptotic effect.57 After treatment of both types of the cancer cell lines with the compounds 3, 4, 8a, 6b, 8b, 9b, 13, 15, 14 and 16 at concentration of 100 µM and (Cs–14) NPs at concentration 100 µg for 24 hrs. Our results showed that there were no much differences in the concentration of DNA between treated and untreated cells for compounds 3, 4, 8a, 6b, 8b and 9b, as shown in Tables 5 and 6, and Figures 8–10. In contrary, the other compounds 13, 15, 14, 16 and (Cs–14) NPs showed a notable alteration in the DNA concentration between the treated and untreated cells (Mcf-7 and Huh-7).

Data are illustrated in (Table 7). There was no significant change in bands shape between cells treated with our selected compounds 3, 4, 8a, 6b, 8b and 9b and the untreated ones (control cell).

**Detection of Caspase-3, Bcl-2 protein levels**

This investigation was performed to evaluate the apoptotic activity of the newly synthesized compounds, the most compound revealed cytotoxic activity on Huh-7 and Mcf-7 cell lines, compound 14 after treatment of cells with 100 µM for 24 hrs. Caspase-3 and Bcl-2 proteins levels were measured and the results showed a marked increase in the Caspase-3 activity on both cell lines compared to the untreated one. In contrast, the level of the anti-apoptotic protein level of Bcl-2 showed marked decrease in the protein levels compared to the untreated cells as shown in Figure 11. These results confirm the apoptotic activity of compound 14.

### Table 1 Cytotoxicity of the synthesized candidates on breast (Mcf-7) and liver (Huh-7) cancer cell lines

| Compound No. | IC50 on Mcf-7 µM | IC50 on Huh-7 µM | IC50 on VERO µM |
|--------------|------------------|------------------|-----------------|
| (3)          | >100             | >100             | >100            |
| (4)          | >100             | >100             | >100            |
| (6a)         | >100             | >100             | >100            |
| (6b)         | >100             | >100             | >100            |
| (7a)         | >100             | >100             | >100            |
| (7b)         | >100             | >100             | >100            |
| (9b)         | >100             | >100             | >100            |
| (11)         | <100             | <100             | <100            |
| (12)         | <100             | <100             | <100            |
| (13)         | 26.66            | 42.9             | 98.57           |
| (14)         | 12.203           | 24.59            | >100            |
| (15)         | 55.026           | 30.67            | >100            |
| (16)         | 14.84            | 26.64            | >100            |
| Paclitaxel   | –                | 4.034            | –               |
| Doxorubicin  | 1.169            | 6.69             | –               |

### Table 2 Cytotoxicity of compound (14) as a nanoformulation loaded on chitosan NPs (Cs–14) NPs on breast (Mcf-7) and liver (Huh-7) cancer cell lines

| Compound No. | IC50 on Mcf-7 µg | IC50 on Huh-7 µg |
|--------------|------------------|-----------------|
| Cs NPs       | <100             | <100            |
| (Cs–14) NPs | 30.68 µg/mL      | 37.19 µg/mL     |

9b against the same cell lines. This indicates that xylose’s derivatives had higher antitumor activities than the corresponding arabinose’s derivatives as shown in Figure 4. Cells treated with compounds 3, 4, 8a, 9b, 6b and 8b showed no significant morphology alteration compared to untreated cells (Figure 5). Whereas the cells treated with compounds 13, 15, 14, 16 and (Cs–14) NPs showed moderate changes in the cell morphology from adherent cells to shrieked/rounded cellular features and smearing at most of the bands as illustrated in Figure 5. Such changes were observed after treatment of either Huh-7 or Mcf-7 with these compounds. These changes might be considered to be hallmark of initial stage of apoptosis.

**Drug combination analysis using median effect principle**

Nature of the combination of two medications was determined by median effect rule based on Chou principle.53 This was performed by plotting the dose-effect curves for each drug in choice and wither constant or nonconstant ratios for each compound as previously demonstrated by Chou and Talalay.54 The combination index indicates whether the two compounds interact in an additive (CI=1), synergistic (CI<1) or antagonistic (CI>1) manner. The median effect analysis and calculation of combination index of free (Cs NPs) and compound 14 using the CompuSyn software showed that (Cs NPs) and compound 14 produced different results with different cell lines.
Figure 4: Representative graph showing survival of Huh-7 and MCF-7 cell lines grown compared to Doxorubicin in the presence of increasing concentrations of compounds 13, 14, 15 and 16.

Figure 5: Morphological examination of cell lines treated with the selected compounds (3, 4, 8a, 6b, 8b, 9b, 13, 14, 15, 16 (Cs–14) NPs) treatment on (A) Huh-7 and (B) MCF-7 cell lines. Light microscopy (phase contrast, magnification power of 400X) 24 hrs exposure 100 μM (high dose from each compound).
promising application as an antitumor drug after further chemical and biological investigations in vivo model.

**Conclusion**

A novel series of pyrazolopyrimidine thioglycosides and pyrazolopyridine thioglycosides were synthesized and their anticancer activities were evaluated. Even most of the newly synthesized products revealed moderate anticancer activity against human liver cancer (Huh-7) and breast cancer (Mcf-7) cell lines. There were some promising compounds which had a high cytotoxic effect on both cancer cell lines (Huh-7 and Mcf-7), compound 14 was the most cytotoxic compound followed by compound 16, 13 and 15. (Cs—14) nanoparticles were successfully prepared by the ionic gelation method, as confirmed by instrumental analytical techniques (FTIR and TEM). The particle size was spherical in shape with a smooth surface, size 105 nm and zeta potential 40.2 mV. Results of anticancer activity via MTT and (Caspase-3 and Bcl-2) assays proved the toxicity of our compounds and (Cs—14) NPs toward breast and liver cancer cell lines. Therefore, our results indicated that the use of Cs—14 nanoparticle system in breast and liver cancer can be used as drug delivery in which the side effects of conventional chemotherapy could be reduced. This was confirmed by DNA fragmentation and determination of protein activity for both Caspase-3 and Bcl-2. This will open further biological research to extensively evaluate the mechanism of antitumor activity of these compounds on biomedical application. Moreover, in vivo studies required to use this system for future applications.

**Experimental Chemistry**

All melting points were measured with a Gallenkamp melting point apparatus. The $^1$H NMR and $^{13}$C NMR spectra were measured on a Jeol-500 MHz spectrometer in DMSO-d$_6$ or CDCl$_3$ using Si(CH$_3$)$_4$ as an internal standard at the National Research Centre, Cairo, Egypt. The IR spectra were recorded on a Pye Unicam Spectra-1000 (KBr disk). Elemental analyses were obtained from the Micro analytical

| Fa | Sample | Compound (14) Dose | (Cs NPs) Dose | (CI) | Type of interaction |
|----|--------|-------------------|---------------|------|-------------------|
| 0.5 | (14)  | 9.31247           | 137.659       | 0.2734 | Synergism |
|     | (Cs NPs) | 0.11007          | 36.6894       |      |                   |
|     | (Cs—14) NPs |        |               |      |                   |
| 0.75 | (14)   | 17.9471          | 184.852       | 1.22373 | Antagonism |
|      | (Cs NPs) | 0.65828          | 219.428       |      |                   |
|      | (Cs—14) NPs |       |               |      |                   |
| 0.9  | (14)   | 34.5879          | 248.222       | 5.40076 | Strong antagonism |
|      | (Cs NPs) | 3.93700          | 1312.33       |      |                   |
|      | (Cs—14) NPs |       |               |      |                   |

| Fa | Sample | Compound (14) Dose | (Cs NPs) Dose | (CI) | Type of interaction |
|----|--------|-------------------|---------------|------|-------------------|
| 0.5 | (14)  | 8.80448           | 137.659       | 0.23807 | Synergism |
|     | (Cs NPs) | 0.09391          | 31.3047       |      |                   |
|     | (Cs—14) NPs |        |               |      |                   |
| 0.75 | (14)   | 45.2704          | 184.852       | 0.96913 | Slight synergism |
|      | (Cs NPs) | 0.53093          | 176.978       |      |                   |
|      | (Cs—14) NPs |       |               |      |                   |
| 0.9  | (14)   | 232.768          | 248.222       | 4.04366 | Strong antagonism |
|      | (Cs NPs) | 3.00158          | 1000.53       |      |                   |
|      | (Cs—14) NPs |       |               |      |                   |

Table 4 Combination index of chitosan and compound (14) Nanocomposite in Mcf-7 cell line and its different cytotoxic effects as calculated by CompuSyn software

Table 3 Combination index of chitosan and compound (14) nanocomposite in Huh-7 cell line and its different cytotoxic effects as calculated by CompuSyn software
Data Center at Cairo University. Progress of the reactions was monitored by TLC using aluminum sheets coated with silica gel F254 (Merck). Viewing under a short-wavelength UV lamp effected detection, biological studies were performed at the National Cancer Institute, Cairo University and at the National Research Center, Dokki, Giza, Egypt.

General procedure for synthesizing compounds 3 and 11

To a solution of 4-aminoantipyrine 1 (2.03 g, 10 mmol) in glacial acetic acid (10 mL), sodium 2,2 dicyanoethene-1,1-bis(thiolate) 2 (1.86 g, 10 mmol) or sodium cyanocarbonimidodithioate 10 (1.62 g, 10 mmol) were added, and warmed for 5 mins. A precipitate was formed, filtered off and recrystallized from the appropriate solvent to give compounds 3, 11.

2-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-ylamino)(mercapto)methylene)malononitrile (3)

Yield: (2.49 g, 80%) as yellow solid, m.p. 190–191°C; IR (KBr, cm⁻¹): ν 3,415 (NH), 3,063 (CH, aromatic) 2,928 (CH₃), 2,217, 2,189 (2CN), 1,653 (CO), 1,615 (C=C); ¹H NMR (500 MHz, DMSO-d₆): δ 2.24 (s, 3H, CH₃), 3.09 (s, 3H, CH₃), 7.48–7.31 (m, 5H, C₆H₅), 9.55 (s, 1H, NH), 13.45 (s, 1H, SH). ¹³C NMR: δ 12.50 (CH₃), 34.00 (CH₃), 61.00 (C=(CN)₂), 110.00 (C-4), 112.45 (CN), 133–123.55 (7C, 6Ar-C, C-5), 161.00 (CO), 163.50 (C=C). Anal. Calcd. For. C₁₅H₁₃N₅OS (311.36): C% 57.86; H% 4.21; N% 22.49; S% 10.30. Found: C% 57.78; H% 4.15; N% 22.36; S% 10.22.

N’-Cyano-N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)carbamimidothioic acid (11)

Yield: (2.30 g, 80%) as yellow solid, m.p. 178–179°C; IR (KBr, cm⁻¹): ν 3,477 (NH), 3,072 (CH), 2,205 (CN), 1,640 (CO), 1,532 (C=N); ¹H NMR (500 MHz, DMSO-d₆): δ 2.13 (s, 3H, CH₃), 3.08 (s, 3H, CH₃), 7.47–7.31 (m, 5H, C₆H₅), 9.41 (s, 1H, NH), 10.90 (s, 1H, SH); ¹³C NMR: δ 12.26 (CH₃), 35.23 (CH₃), 117.45 (C-4), 164.26 (CO), 166.42 (C=N). Anal. Calcd. For. C₁₃H₁₃N₅OS (287.34): C% 57.86; H% 4.21; N% 22.49; S% 10.30. Found: C% 57.87; H% 4.15; N% 22.36; S% 10.22.

Figure 6 The simulated (A) Dose Effect Curve (B) Median-effect Plot (C) Combination Index Plot (D) DRI Plot (Cs–14) NPs on Huh-7 cell line calculated by CompuSyn Software.
54.34; H% 4.56; N% 24.37; S% 11.16. Found: C% 54.22; H% 4.44; N% 24.22; S% 11.11.

**General procedure for synthesizing compounds 4 and 12**

As a solution of 3 or 11 (3.11 or 2.87 g, 10 mmol) in ethanol (20 mL) was treated with concentrated hydrochloric acid (1 mL), the reaction mixture was heated under reflux for 30 mins, and then evaporated under reduced pressure. The resulting solid product was collected by filtration and recrystallized from methanol to give 4 and 12.

**7-Imino-5-mercapto-1,7a-dimethyl-3-oxo-2-phenyl-2,3,7,7a-tetrahydro-1H-pyrazolo[4,3-b]pyridine-6-carbonitrile (4)**

Yield: (2.43 g, 78%) as yellow solid, m.p. 240–242°C (MeOH); IR (KBr, cm⁻¹): ν 3,299 (NH), 3,925 (CH₃), 2,211

**Table 5** Genomic DNA content of DNA fragmentation assay after treatment of Mcf-7 cells with compounds (CC, 3, 4, 8a, 6b, 8b, 9b) at high concentration (100 μM) of each compound after 24 hrs of cell exposure

| Lane No. | Sample | DNA Conc. (ng/μL) |
|----------|--------|-------------------|
| 1        | Control | 42.7              |
| 2        | 3       | 44.6              |
| 3        | 4       | 37.4              |
| 4        | 8a      | 42.4              |
| 5        | 6b      | 54.8              |
| 6        | 8b      | 43.2              |
| 7        | 9b      | 42.3              |

| Lane No. | Sample | DNA Conc. (ng/μL) |
|----------|--------|-------------------|
| 1        | Control | 35.6              |
| 2        | 3       | 28.1              |
| 3        | 4       | 27.2              |
| 4        | 8a      | 30.5              |
| 5        | 6b      | 28.9              |
| 6        | 8b      | 27.6              |
| 7        | 9b      | 33.3              |

**Table 6** Genomic DNA content of DNA fragmentation assay after treatment of Huh-7 cells with compounds (CC, 3, 4, 8a, 6b, 8b, 9b) at high concentration (100 μM) of each compound for 24 hrs of cell exposure

Figure 7 The simulated (A) Dose Effect Curve (B) Median-effect Plot (C) Combination Index Plot (D) DRI Plot (Cs–14) NPs on Mcf-7 cell line calculated by CompuSyn Software.
Figure 8 EB – stained gel electrophoresis of genomic DNA extraction at high concentration 300 ng from untreated and treated MCF-7 cell line with Lane 1: control, lane 2: 100 bp ladder, lane 3: compound 3, lane 4: compound 4, lane 5: compound 8a, lane 6: compound 6b, lane 7: compound 8b, lane 8: compound 9b with high concentration (100 µM) of each compound.

Figure 9 EB – stained gel electrophoresis of genomic DNA extraction at high concentration 300 ng from untreated and treated Huh-7 cell line with Lane 1: control, lane 2: 100 bp ladder, lane 3: compound 3, lane 4: compound 4, lane 5: compound 8a, lane 6: compound 6b, lane 7: compound 8b, lane 8: compound 9b with high concentration 100 µM of each compound.

Table 7 Genomic DNA content of DNA fragmentation assay after treatment of Huh-7 and MCF-7 cells with compounds (CC, 13, 15, 14, 16 (CS–14) NPs) at high concentration (100 µM) of each compound for 24 hrs of cell exposure

| Lane No. | Sample | DNA Conc. (ng/µL) |
|----------|--------|------------------|
| 1        | Ladder | 100              |
| 2        | Control Huh-7 | 45.8         |
| 3        | 13      | 20.4             |
| 4        | 15      | 16               |
| 5        | 14      | 27.3             |
| 6        | 16      | 36.6             |
| 7        | (Chitosan–14) NPs with conc. 100 µg on Huh-7 | 29.3 |
| 8        | Ladder | 50               |
| 9        | Control MCF-7 | 42.5          |
| 10       | 13      | 22.5             |
| 11       | 15      | 24               |
| 12       | 14      | 14               |
| 13       | 16      | 19.7             |
| 14       | (Chitosan–14) NPs with conc. 100 µg on MCF-7 | 17  |
| 15       | Ladder | 50               |

7-Imino-5-mercaptopo-1,7a-dimethyl-2-phenyl-7,7a-dihydro-1H-pyrazolo[4,3-d]pyrimidin-3(2H)-one (12)

Yield: (2.24 g, 78%) as yellow solid, mp 225–227°C (MeOH); IR (KBr, cm⁻¹): ν 3,329 (NH), 3,025 (CH aromatic), 2,973 (CH₃), 1,642 (CO); ¹H NMR (500 MHz, DMSO-d₆): δ 2.04 (s, 3H, CH₃), 3.01 (s, 3H, CH₃), 6.21 (s, br, 1H, =NH), 7.37–7.28 (m, 5H, C₆H₅), 12.46 (s, 1H, SH); ¹³C NMR: δ 12.13 (CH₃), 40.42 (N-CH₃), 61.47 (C-7a), 138.21–124.22 (6C, Ar-C), 161.23 (CO), 163.64 (C-3a), 165.24 (C-7), 176.28 (C-5). Anal. Calcd. for C₁₃H₁₃N₃O₅S (311.36): C% 57.86; H% 4.21; N% 22.49; S% 10.30. Found: C% 57.77; H% 4.10; N% 22.36; S% 10.24.

General procedure for synthesizing compounds 6a,b

To a solution of 3 (3.11 g, 10 mmol) in aqueous KOH [0.56 g (0.01 mol) in 6 mL of distilled water], a solution of 2,3,5 tri-O-acetyl-α-D-xylo- or arabino-furanosyl bromide 5a,b (4.52 g, 0.011 mol) in acetone (30 mL) was added. The reaction mixture was stirred at room temperature until the reaction was judged complete by TLC (1–3 hrs), using chloroform: ether 4:1, v/v (Rf 0.66–0.70 region), then evaporated under reduced pressure at 60°C and the residue was washed with...
2-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-ylamino)(2,3,5-tri-O-acetyl-β-D-xylofuranosylthio)methylene) malononitrile (6a)

Yield: (3.99 g, 70%) as yellow solid, m.p. 216–217°C (EtOH); IR (KBr, cm⁻¹): ν 3395 (NH), 3028 (CH aromatic), 2972 (C=O exch., 1H, NH). Anal. Calcd. For Chemical Formula: C₆₀H₈₂N₂O₈S: C% 54.83; H% 4.80; N% 12.25; S% 5.55. Found: C% 54.74; H% 4.78; N% 12.30; S% 5.63. General procedure for synthesizing compound 13

To a solution of 11 (2.87 g, 10 mmol) in aqueous KOH [0.56 g (0.01 mol) in 6 mL of distilled water], a solution of N-(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl) carbamimidothioate (13) in acetone (30 mL) was added. The reaction mixture was stirred at room temperature until the reaction was judged complete by TLC (1–3 hrs), using chloroform: ether 4:1, v/v (Rf 0.66–0.70 region), then evaporated under reduced pressure at 60°C and the residue was washed with distilled water to remove KBr. The product was dried prior to crystallization from methanol to give compound 13.

2-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-ylamino)(2,3,4-tri-O-acetyl-β-D-arabinofuranosylthio)methylene) malononitrile (6b)

Yield: (4.27 g, 75%) as yellow solid, m.p. 206–207°C (EtOH); ¹H NMR (500 MHz, DMSO-d₆): δ 1.98–2.11 (3s, 9H, 3xOAc), 2.27 (s, 3H, CH₃), 3.41 (s, 3H, CH₃), 4.35–4.36 (m, 2H, 2H-5'), 4.52–4.53 (m, 1H, H-4'), 4.83 (t, J=9.8 Hz, 1H, H-3'), 5.14 (t, J=10.3 Hz, 1H, H-2'), 5.33 (d, J=6.5 Hz, 1H, H-1'), 7.34–7.67 (m, 5H, C₆H₅), 11.56 (s, D₂O exch., 1H, NH). Anal. Calcd. For Chemical Formula: C₇₀H₈₀N₂O₈S: C% 54.83; H% 4.78; N% 12.30; S% 5.63. Found: C% 54.75; H% 4.74; N% 12.25; S% 5.55.
5), 161.63 (CO), 166.29 (-C=N), 172.24 (4CO). Anal. Calcd. For C_{22}H_{27}N_{2}O_{3}S (545.56): C% 52.84; H% 4.99; N% 12.84; S% 5.88. Found: C% 52.75; H% 4.82; N% 12.66; S% 5.80.

General procedure for synthesizing compounds 7a,b
To a solution of 4 (3.11 g 10 mmol) in aqueous KOH [0.56 g (0.01 mol) in 6 mL of distilled water], a solution of 2,3,5-tri-O-acetyl-a-D-xylo- or arabino-furanosyl bromide 5a,b (0.011 mol) in acetone (30 mL) was added. The reaction mixture was stirred at room temperature until the reaction was judged complete by TLC (1–3 hrs), using chloroform: ether 4:1, v/v (Rf 0.66–0.70 region), then evaporated under reduced pressure at 40°C and the residue was washed with distilled water to remove KBr. The product was dried prior to crystallization from methanol to give compounds 7a,b.

7-Imino-1,7a-dimethyl-5-(2,3,4-tri-O-acetyl/-β-D-xylofuranosyothio)-3-oxo-2-phenyl-2,3,7a-tetrahydro-1H-pyrrazolo[4,3-b]pyridine-6-carbonitrile (7a)
Yield: (4.50 g, 79%) as yellow solid, m.p. 208–209°C (EtOH); IR (KBr, cm⁻¹): ν 3,394 (NH), 3,035 (CH, aromatic), 2,952 (CH), 2,210 (CN), 1,743 (4CO), 1,638 (CO), 1,592 (C=C=N); ¹H NMR (500 MHz, DMSO-d₆): δ 1.97–2.12 (3s, 9H, 3xOAc), 2.36 (s, 3H, CH₃), 2.62 (s, 3H, CH₃), 4.13–4.19 (m, 3H, 2H-5'), 4.36–4.37 (m, 1H, H-4'), 4.62 (t, J = 9.3 Hz, 1H, H-3'), 4.98 (t, J = 9.1 Hz, 1H, H-2'), 5.56 (d, J₁=J₂ =10.6 Hz, 1H, H-1'), 6.64 (s, D₂O exh., 1H, NH), 7.34–7.76 (m, 5H, C₆H₅); ¹³C NMR: δ 19.16 (CH₃), 21.36 (3CH₃CO), 38.95 (CH₃), 51.24 (C-7a), 62.11 (C-5'), 69.27 (C-4'), 71.86 (C-3'), 73.75 (C-2'), 79.58 (C-1'), 82.31 (C-6), 116.38 (CN), 125.21–139.51 (6C, Ar-C), 159.34 (CO), 162.41 (C-7), 164.11 (C-3a), 165.14 (C-7), 167.22 (C-5), 171.42 (4CO). Anal. Calcd. For C_{22}H_{27}N_{2}O_{3}S (569.59): C% 54.83; H% 4.78; N% 12.30; S% 5.63. Found: C% 54.75; H% 4.70; N% 12.26; S% 5.54.

General procedure for synthesizing compound 14
To a solution of 12 (2.87 g, 10 mmol) in aqueous KOH [0.56 g (0.01 mol) in 6 mL of distilled water], a solution of 2,3,5-tri-O-acetyl-α-D-xylo-furanosyl bromide 5a (0.011 mol) in acetone (30 mL) was added. The reaction mixture was stirred at room temperature until the reaction was judged complete by TLC (1–3 hrs), using chloroform: ether 4:1, v/v (Rf 0.66–0.70 region), then evaporated under reduced pressure at 40°C and the residue was washed with distilled water to remove KBr. The product was dried prior to crystallization from methanol to give compound 14.

7-Imino-1,7a-dimethyl-5-(2,3,5-tri-O-acetyl-/β-D-xylofuranosyothio)-2-phenyl-7,7a-dihydro-1H-pyrrazolo[4,3-d] pyrimidin-3(2H)-one (14)
Yield: (4.32 g, 70%) as yellow solid, m.p. 222–223°C (EtOH); IR (KBr, cm⁻¹): ν 3,436 (NH), 3,028 (CH, aromatic), 2,925 (CH), 1,751 (CO), 1,653 (CO); ¹H NMR (500 MHz, DMSO-d₆): δ 1.98–2.09 (3s, 9H, 3xOAc), 2.36 (s, 3H, CH₃), 2.95 (s, 3H, CH₃), 4.02–4.13 (m, 2H, 2H-5'), 4.37–4.45 (m, 1H, H-4'), 4.62 (t, J = 9.2 Hz, 1H, H-3'), 5.04 (t, J = 8.6 Hz, 1H, H-2'), 5.53 (d, J₁=J₂ =10.4 Hz, 1H, H-1'), 5.66 (s, br, D₂O exh., 1H, NH), 7.21–7.58 (m, 5H, C₆H₅); ¹³C NMR: δ 18.41 (CH₃), 22.63 (3CH₃CO), 39.68 (CH₃), 55.31 (7a), 62.63 (C-5'), 68.74 (C-4'), 72.64 (C-3'), 74.22 (C-2'), 78.81 (C-1'), 124.46–143.21 (6C, Ar-C), 161.47 (CO), 166.83 (3a), 169.33 (C-7), 173.19 (4CO), 178.52 (C-5). Anal. Calcd. For C_{22}H_{27}N_{2}O_{3}S (545.56): C% 52.84; H% 4.99; N% 12.84; S% 5.88. Found: C% 52.69; H% 4.86; N% 12.77; S% 5.80.

General procedure for deacetylation of compounds 8a,b and 15
Dry gaseous ammonia was passed through a solution of protected nucleosides 6a,b or 13 (6.41 g or 6.17 g, 10 mmol) in dry methanol (20 mL) for 10 mins with cooling and stirring, then the reaction mixture was stirred at room temperature until the reaction was judged complete by TLC (9–10 hrs) using (CHCl₃/MeOH 9:1) (Rf 0.56–0.58). The resulting mixture was concentrated under reduced pressure to afford a solid...
residue which washed several times by boiling chloroform.
The residue was dried, purified by column chromatography
using chloroform/methanol (9:1) and crystallized from the
appropriate solvent to give compound 8a,b and 15.

2-((1,5-Dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-
4-ylamino)(β-D-xylofuranosylthio)methylene)malononitrile
(8a)

Yield: (3.22 g, 68%) as yellow solid, m.p. 211–212°C (EtOH);
IR (KBr, cm⁻¹): ν 3,532 (OH), 3,413 (NH), 3,069 (CH
aromatic), 2,216 (CN), 2,210 (CN), 1,642 (CO); ¹H NMR
(500 MHz, CDCl₃) δ 2.26 (s, 3H, CH₃), 3.14 (s, 3H, CH₃),
3.53–5.54 (m, 2H, H-5), 3.86–3.87 (m, 2H, H-4), 4.01 (t,
J _1H-2H_ = 8.4 Hz, 1H, H-3), 4.33 (t, J _1H-2H_ = 9.1 Hz,
1H, H-2'), 4.99 (s, D₂O exch., 1H, 5'-OH), 5.15–
5.245 (m, D₂O exch., 2H, 2'-OH, 3'-OH), 6.72 (s, D₂O exch.,
1H, NH), 7.06–7.36 (m, 5H, C₆H₅); ¹³C NMR: δ 28.28 (CH₃),
35.84 (CH₃), 62.36 (C-5'), 69.27 (C-4'), 71.52 (C-3'), 74.51 (C-2'),
83.43 (C-1'), 118.22 (CN), 120.31 (C-4), 124.37–135.72
(6C, Ar-C, C-5), 164.42 (CO), 165.61 (C=O). Anal. Calcd.
For C₂₈H₂₇N₅O₆S (419.45): C% 51.54; H% 5.05; N% 16.70;
S% 7.64. Found: C% 51.46; H% 5.10; N% 16.62; S% 7.52.

**General procedure for synthesizing compounds 9a,b
and 16**

Dry gaseous ammonia was passed through a solution of
protected nucleoside 7a,b or 14 (5.70 or 5.46 g, 10 mmol)
in dry methanol (20 mL) for 10 mins with cooling and
stirring, then the reaction mixture was stirred at room
temperature until the reaction was judged complete by
TLC (9–10 hrs) using (CHCl₃/MeOH 9:1) (RF, 0.56–
0.58). The resulting mixture was concentrated under reduced
pressure to afford a solid residue which washed
several times with boiling chloroform. The residue was
dried, purified by column chromatography using chloro-
form/methanol (9:1) and crystallized from appropriate solvent
to give compounds 9a,b and 16.

7-Imino-1,7a-dimethyl-5-(β-D-xylofuranosylthio)-3-oxo-2-
phenyl-2,3,7,7a-tetrahydro-1H-pyrazolo[4,3-b]pyridine-6-
carboxonitrile(9a)

Yield: (3.30 g, 70%) as yellow solid, m.p. 192–193°C (EtOH);
IR (KBr, cm⁻¹): ν 3,532 (OH), 3,439 (NH), 3,078 (CH
aromatic), 1,649 (CO), 1,599 (C=O); ¹H NMR (500 MHz,
CDCl₃) δ 2.16 (s, 3H, CH₃), 3.11 (s, 3H, CH₃), 3.78–3.79
(m, 2H, H-5'), 3.92–3.94 (m, 1H, H-4'), 4.66 (d, J _1H-2H_ = 8.7 Hz,
1H, H-2'), 4.66 (d, J _1H-2H_ = 10.2 Hz, H-1'), 4.91 (s, D₂O exch.,
1H, 5'-OH), 5.22–5.23 (m, D₂O exch., 2H, 2'-OH and 3'-OH),
7.35–7.86 (m, 5H, C₆H₅); ¹³C NMR: δ 20.47 (CH₃),
41.24 (CH₃), 51.49 (7a), 62.41 (C-5'), 69.27 (C-4'), 71.62 (C-3'),
74.92 (C-2'), 81.93 (C-1'), 86.25 (C-6), 117.44 (CN),
123.68–140.37 (6C, Ar-C), 160.61 (CO), 168.15(3a), 169.61
(C-7), 170.13 (C-5). Anal. Calcd. For C₂₈H₂₇N₅O₆S (443.48):
C% 54.17; H% 4.77; N% 15.79; S% 7.23. Found: C% 54.15;
H% 4.64; N% 15.68; S% 7.16.

7-Imino-1,7a-dimethyl-5-(β-D-arabinofuranosylthio)-3-
oxo-2-phenyl-2,3,7,7a-tetrahydro-1H-pyrazolo[4,3-b]

pyridine-6-carbonitrile (9b)
Yield: (3.20 g, 72%) as yellow solid, m.p. 200–202°C (EtOH); IR (KBr, cm⁻¹) ν 3,545 (OH), 3,436 (NH), 3,062 (CH aromatic), 1649 (CO), 1,600 (C=NR); ¹H NMR (500 MHz, CDCl₃) δ 2.41 (s, 3H, CH₃), 3.14 (s, 3H, CH₃), 3.42–3.45 (m, 2H, 2H-5’), 3.55–3.58 (m, 1H, H-4’), 3.76 (t, J=8.8 Hz, 1H, H-3’), 3.89 (t, J=6.9 Hz, 1H, H-2’), 4.62 (d, 1H, J₁₂ =6.62 Hz, H-1’), 4.93 (s, 3D₂O exch., 1H, 5’-OH), 5.21–5.24 (m, 2D₂O exch., 2H, 2’-OH and 3’-OH), 5.53 (s, 3D₂O exch., 1H,NH), 7.47–7.86 (m, 5H, C₆H₅). Anal. Calcd. For C₃₀H₃₆N₂O₂S (712.56): C% 55.14; H% 4.72; N% 15.71; S% 7.15.

7-lmino-1,7a-dimethyl-5-(β-D-xylofuranosylthio)-2-phenyl-7a,7a-dihydro-1H-pyrazolo[4,3-d]pyrimidin-3(2H)-one (16)
Yield: (3.14 g, 70%) as yellow solid m.p. 188–189°C (EtOH); IR (KBr, cm⁻¹) ν 3,547 (OH), 3,436 (NH), 3,088 (CH aromatic), 1,641 (CO), 1,593 (C=NR); ¹H NMR (500 MHz, CDCl₃) δ 1.63 (s, 3H, CH₃), 2.46 (s,3H, CH₃), 3.63–3.65 (m, 2H, H-5’), 4.24-4.27 (m, 1H, H-4’), 4.46 (t, J=6.4 Hz, 1H, H-3’), 4.55 (t, J=9.2 Hz, 1H, H-2’), 4.91 (s, 3D₂O exch., 1H, 5’-OH), 5.01–5.12 (m, 3D₂O exch., 2H, 2’-OH and 3’-OH), 5.24–5.25 (1H, J₁₂ =10.23 Hz, H-1’), 5.52 (s, 3D₂O exch., 1H,NH), 7.12–7.51 (m, 5H, C₆H₅); ¹³C NMR: δ 17.48 (CH₃), 38.26 (CH₃), 53.64 (7a), 62.33 (C-5’), 67.93 (C-4’), 68.98 (C-3’), 73.96 (C-2’), 81.25 (C-1’), 124.34–136.45 (6C, Ar-C), 161.28 (CO), 164.26 (3a), 166.64 (C-7), 179.15 (C-5). Anal. Calcd. For C₁₉H₁₉N₂O₂S (419.45): C% 51.54; H% 5.05; N% 16.70; S% 7.64. Found: C% 51.46; H% 5.15; N%16.64; S% 7.60%

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Disclosure
The authors report no conflicts of interest in this work.

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