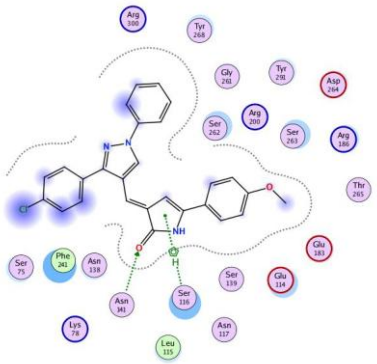
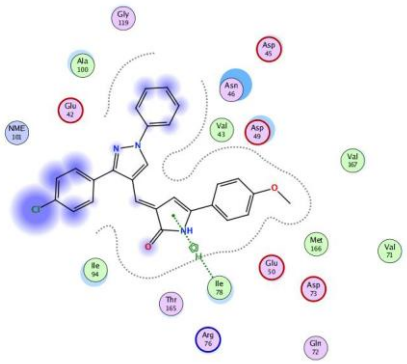
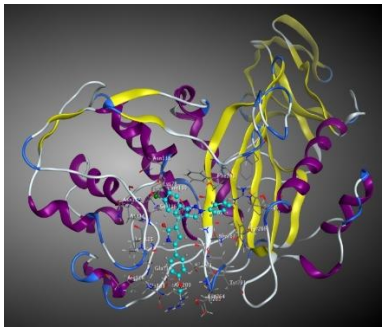
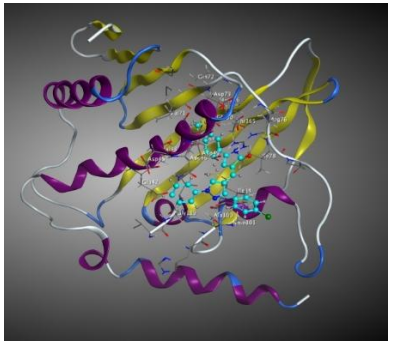
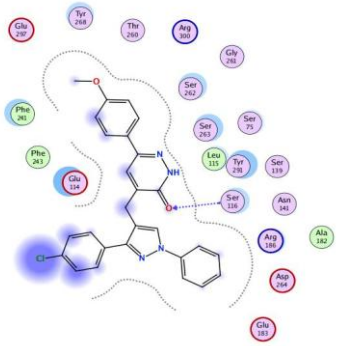
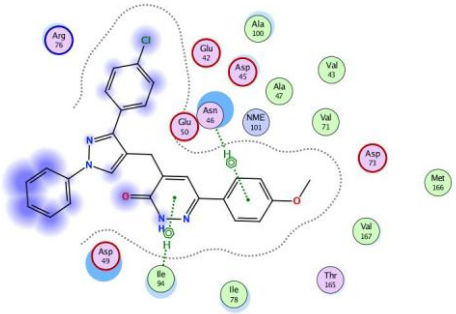
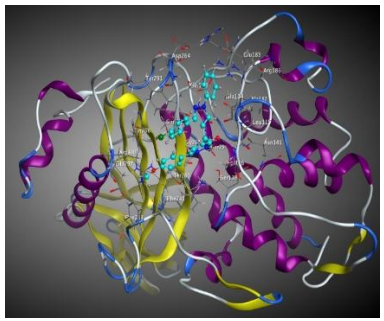
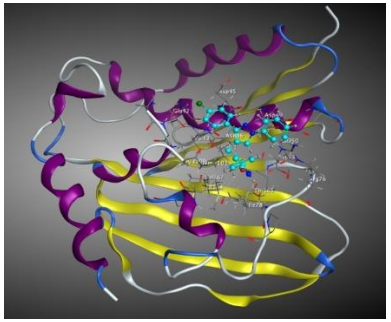
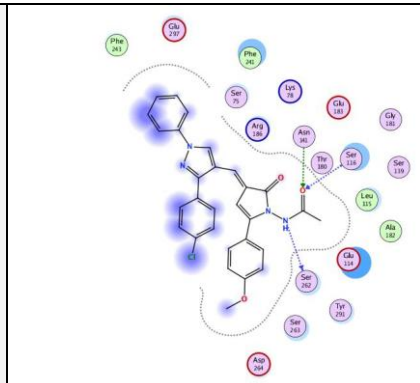
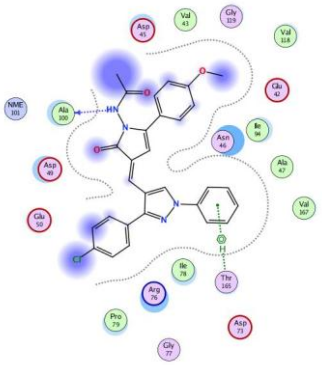
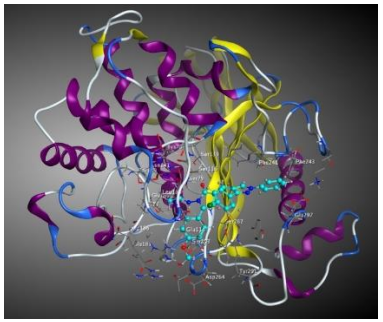
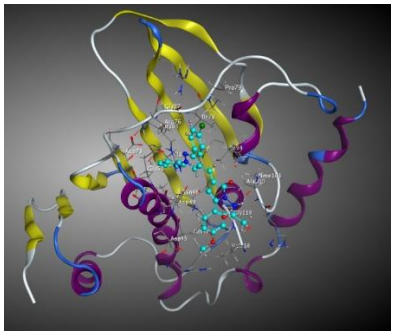
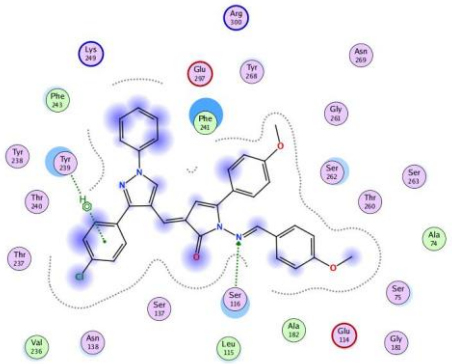
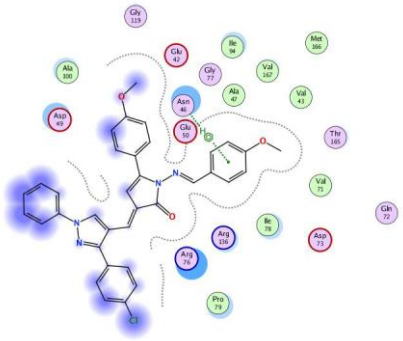
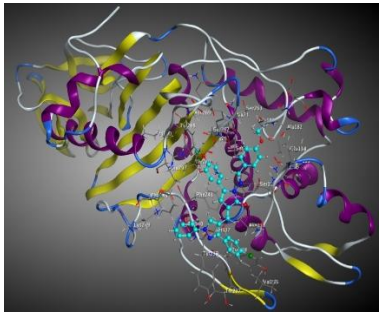
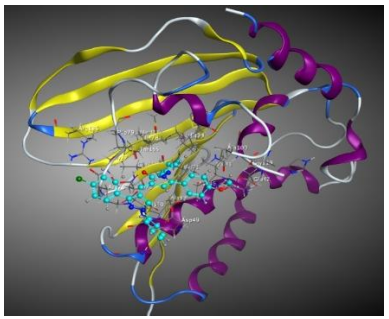
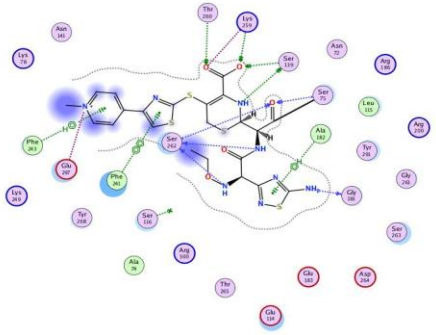
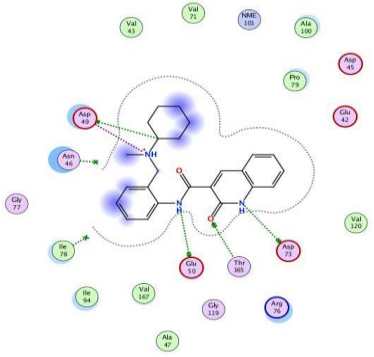
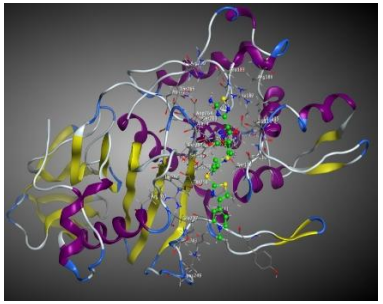
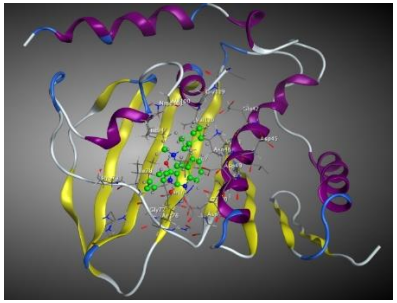
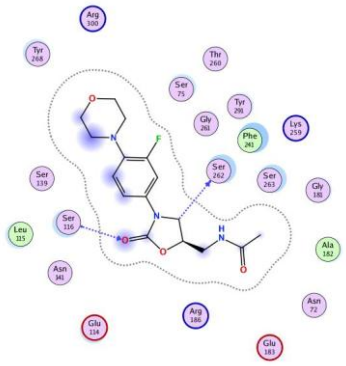
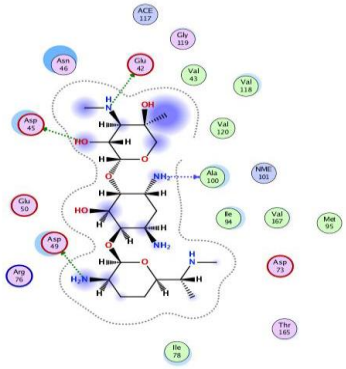
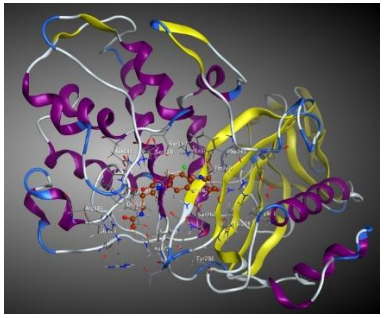
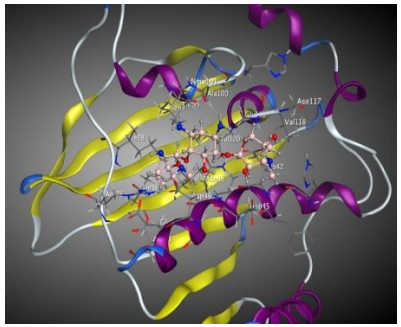
Supporting information

Table S1. 2D and 3D interactions of compounds (**4**, **6**, **7**, and **10**) with Wild-type *S. aureus* PBP4 (PDB ID: 5TW8) and DNA gyrase B (PDB ID: 6KZV) binding pockets compared to co-crystallized ligands (AI8 and E0F) and reference agents (Linezolid and Gentamicin).

Compds.	2D interactions		3D interactions*	
	5TW8	6KZV	5TW8	6KZV
4				
6				

7				
10				
Co-crystallized ligand				

Reference agent				

*The synthesized compounds were colored in cyan, and co-crystallized ligands were colored in green, while reference agents were colored in orange.

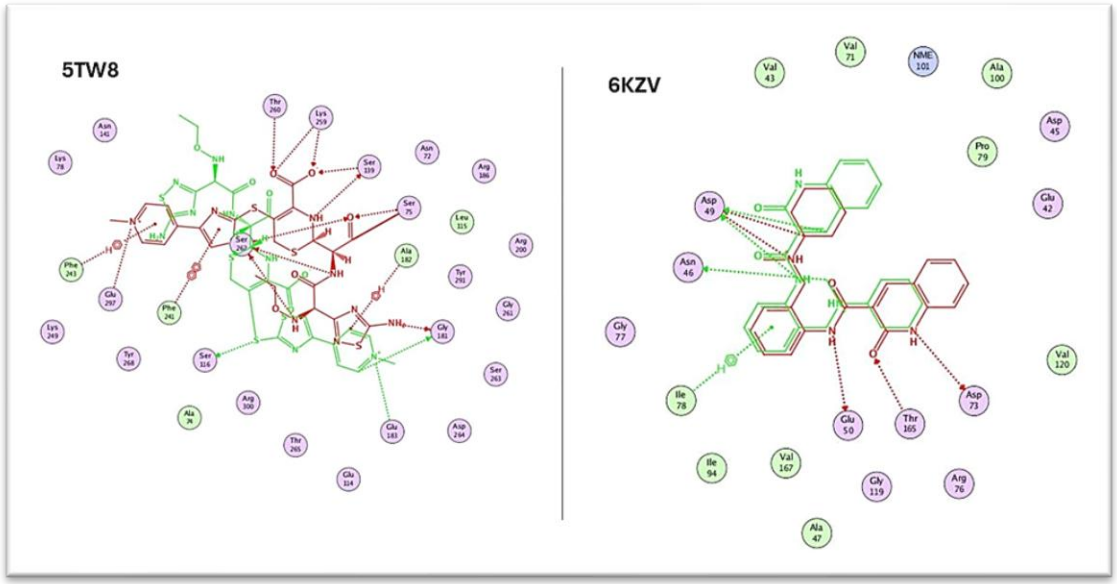


Fig. S1. 2D diagrams of the superimposition of the native and redocked co-crystallized ligands (AI8 ‘left’ and E0F ‘right’) structures at protein targets (PDB ID: 5TW8 and 6KZV) with RMSD values of 1.4939 and 1.1922 Å, respectively.

Table S2. Violations of the synthesized compounds towards pharmacokinetics rules and filters.

Compds.	Lipinski #violations	Ghose #violations	Veber #violations	Egan #violations	Muegge #violations	PAINS #alerts	Brenk #alerts	Leadlikeness #violations
3	1	1	0	1	1	1	1	2
4	0	1	0	0	1	1	1	2
5	0	2	0	0	0	0	3	3
6	1	1	0	0	1	0	0	2
7	1	2	0	0	1	1	1	2
8	2	3	0	1	1	1	1	3
9	1	2	0	0	1	1	2	3
10	2	3	0	1	1	1	2	3

Table S3. Inhibition of the synthesized compounds against CYP isoenzymes.

Compds.	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
3	No	Yes	Yes	No	No
4	No	Yes	Yes	No	No
5	No	Yes	Yes	No	No
6	No	Yes	Yes	No	Yes
7	No	Yes	Yes	No	No
8	No	Yes	Yes	No	No
9	No	Yes	Yes	No	No
10	No	No	No	No	No

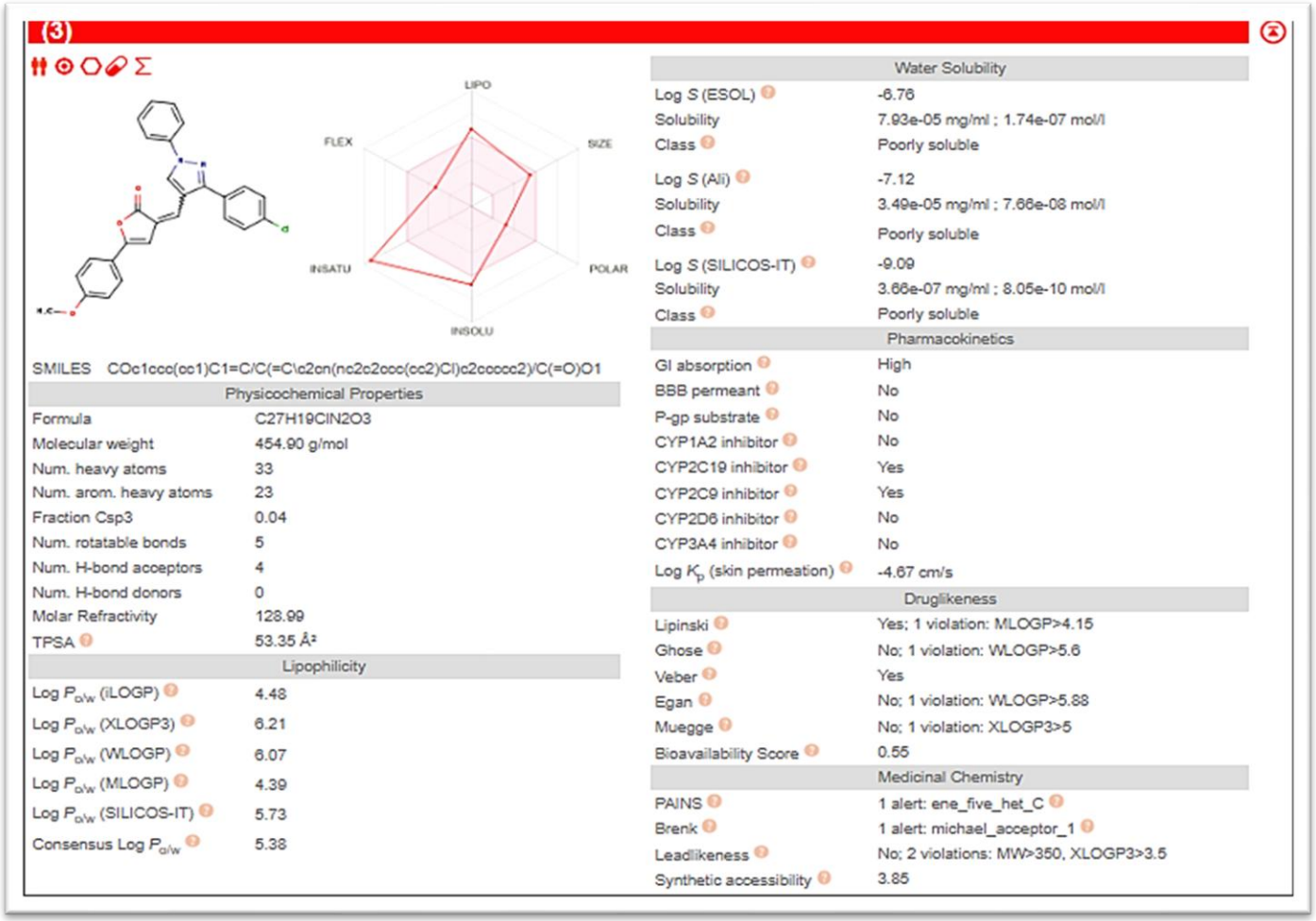


Fig. S2. ADME profile of compound 3.

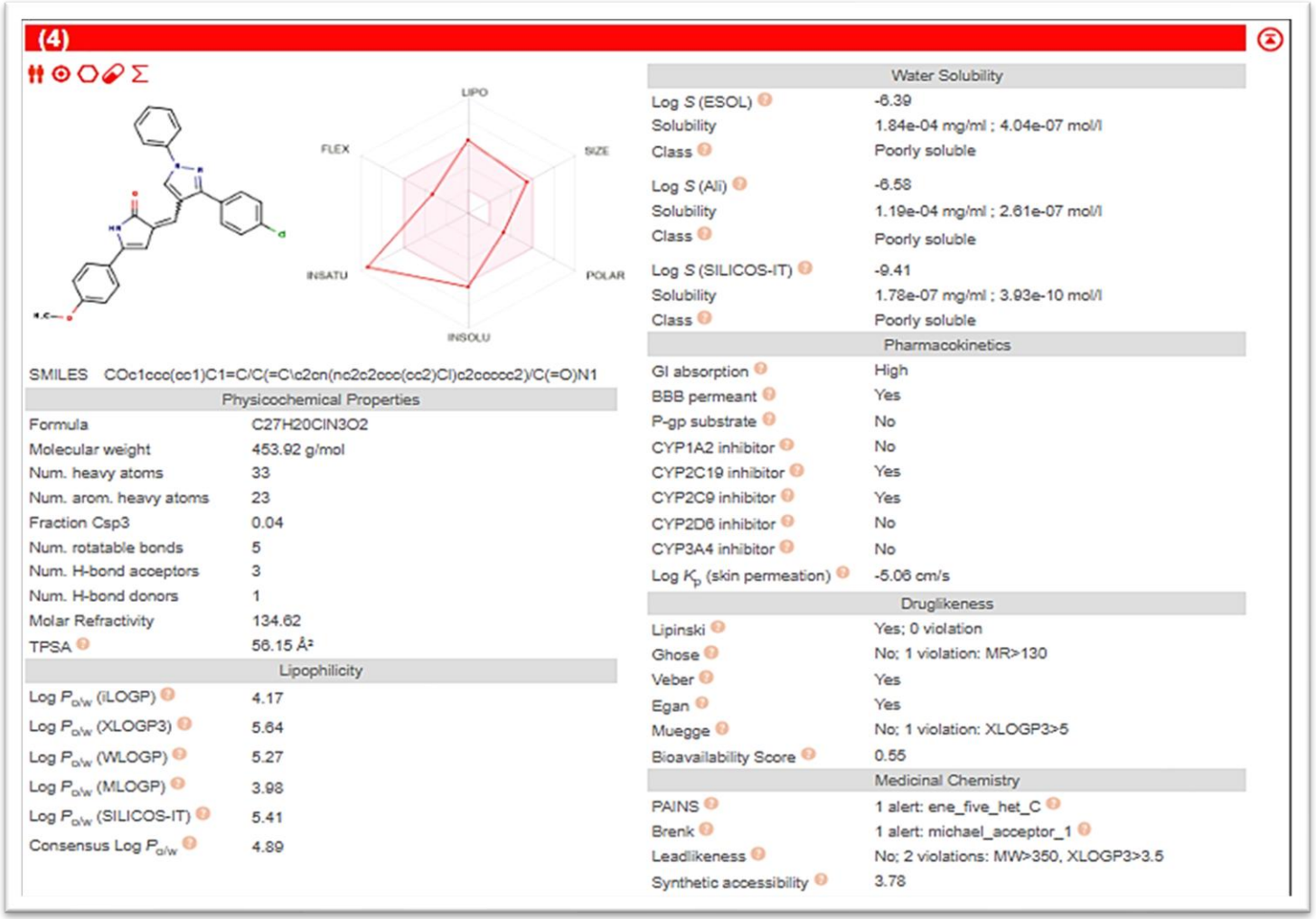


Fig. S3. ADME profile of compound 4.

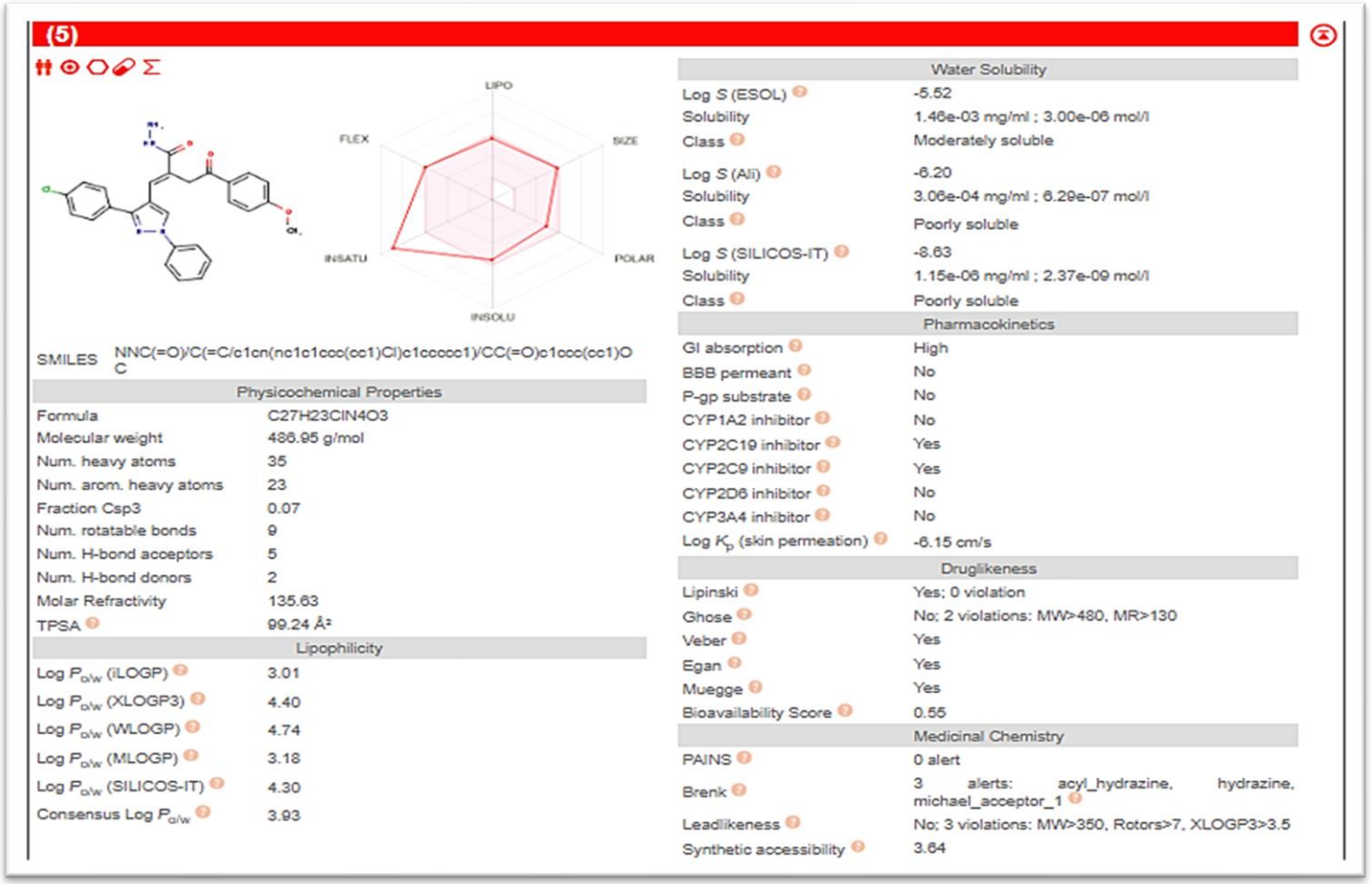


Fig. S4. ADME profile of compound 5.

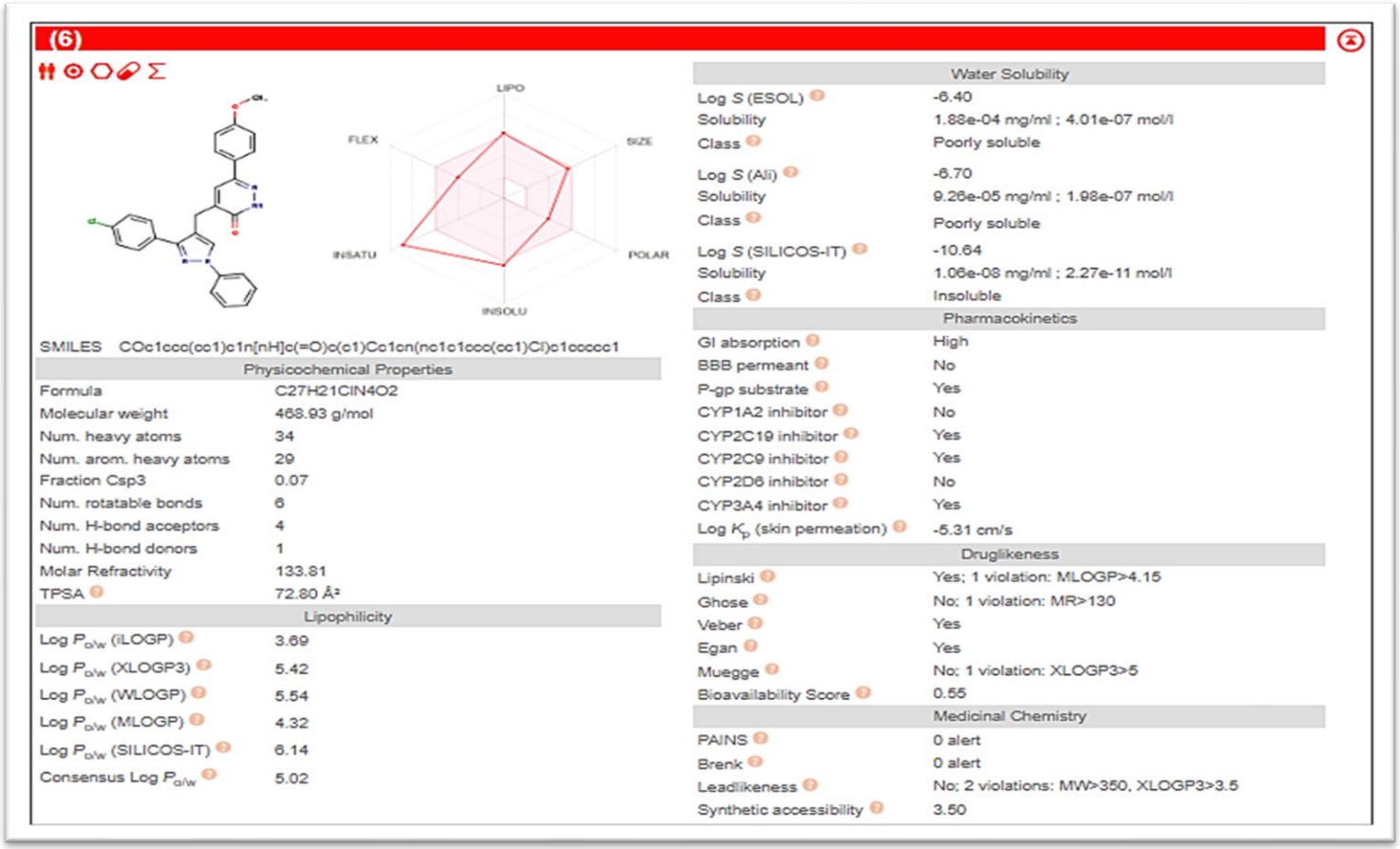


Fig. S5. ADME profile of compound 6.

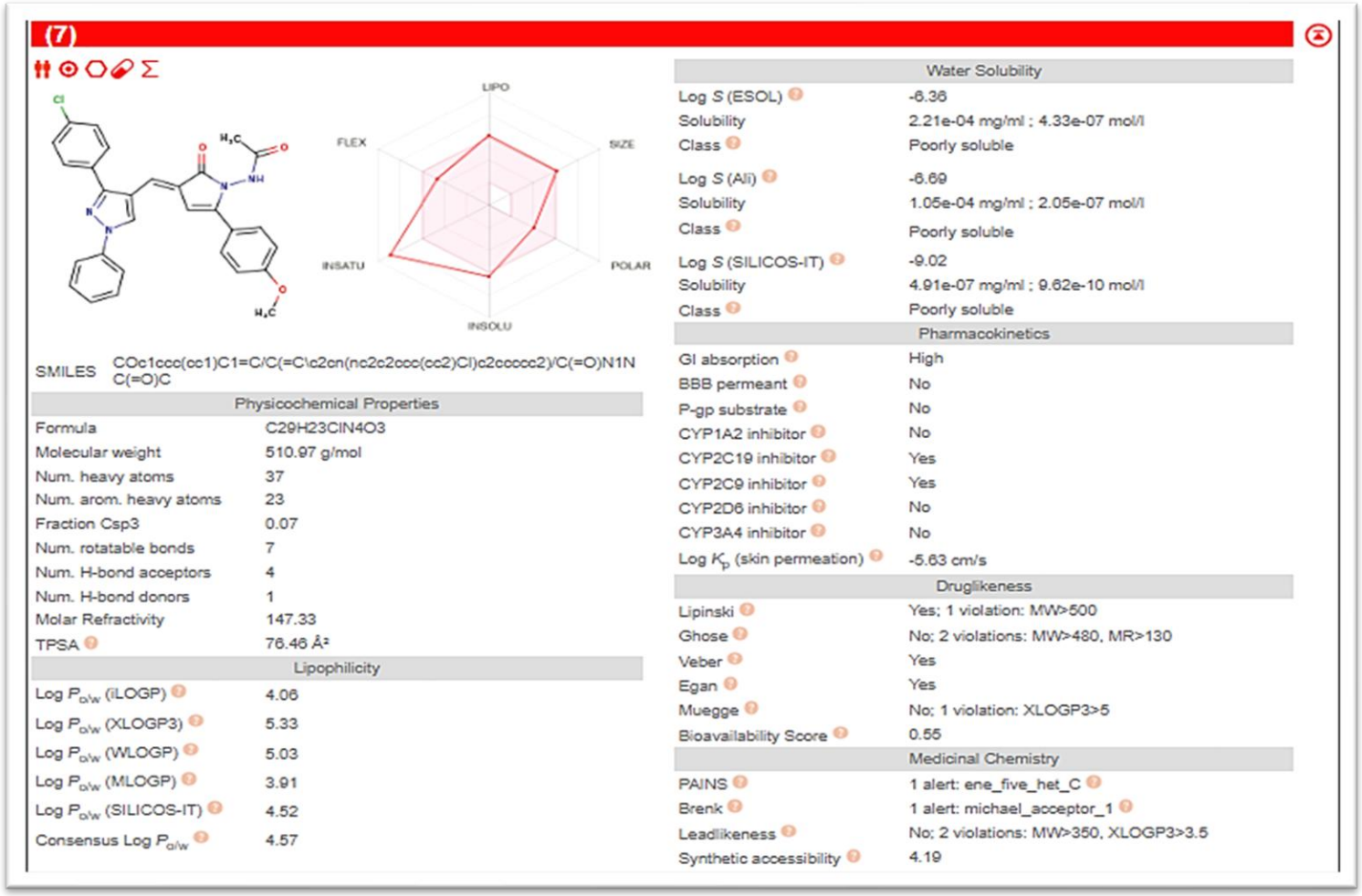


Fig. S6. ADME profile of compound 7.

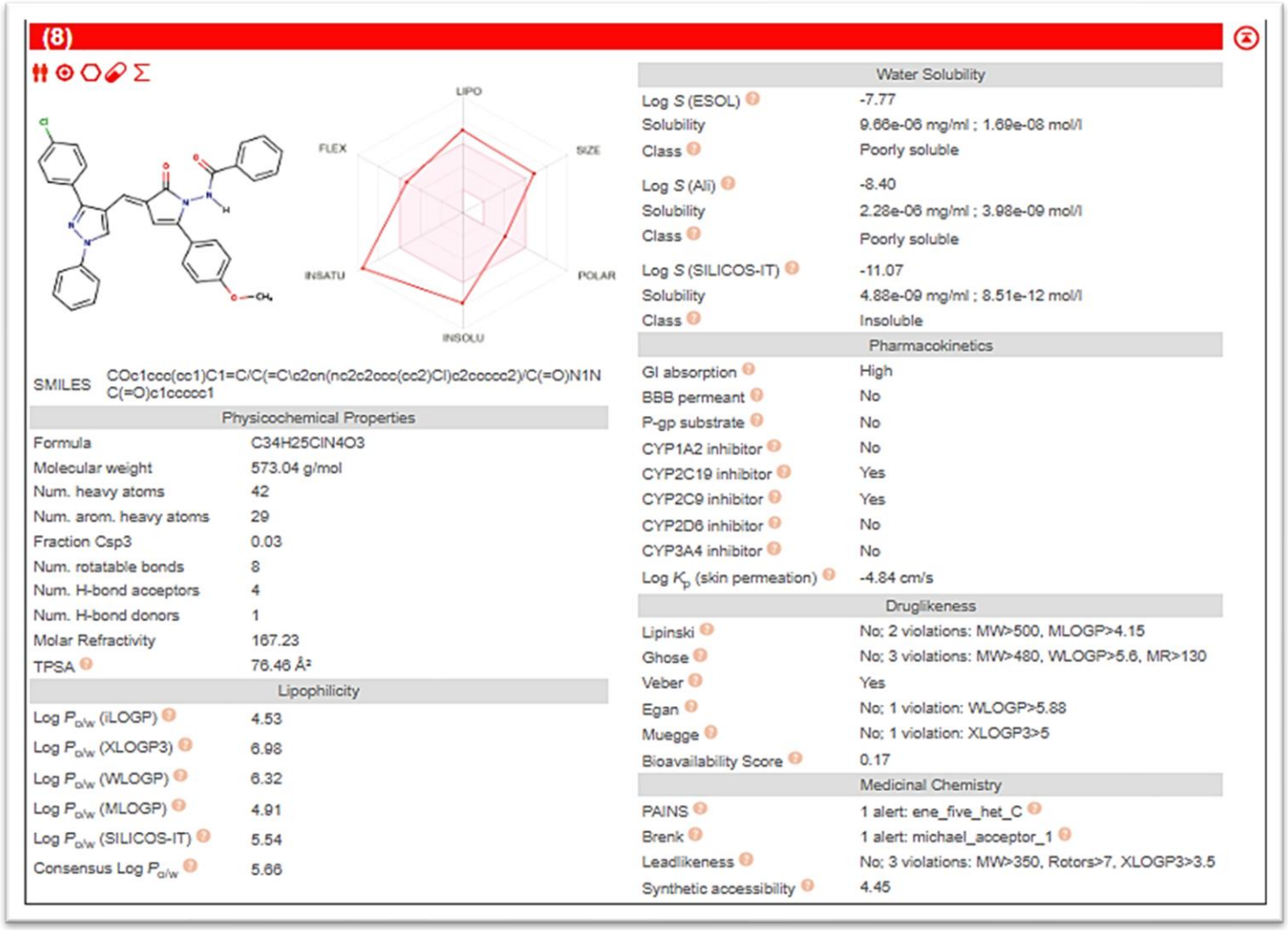


Fig. S7. ADME profile of compound 8.

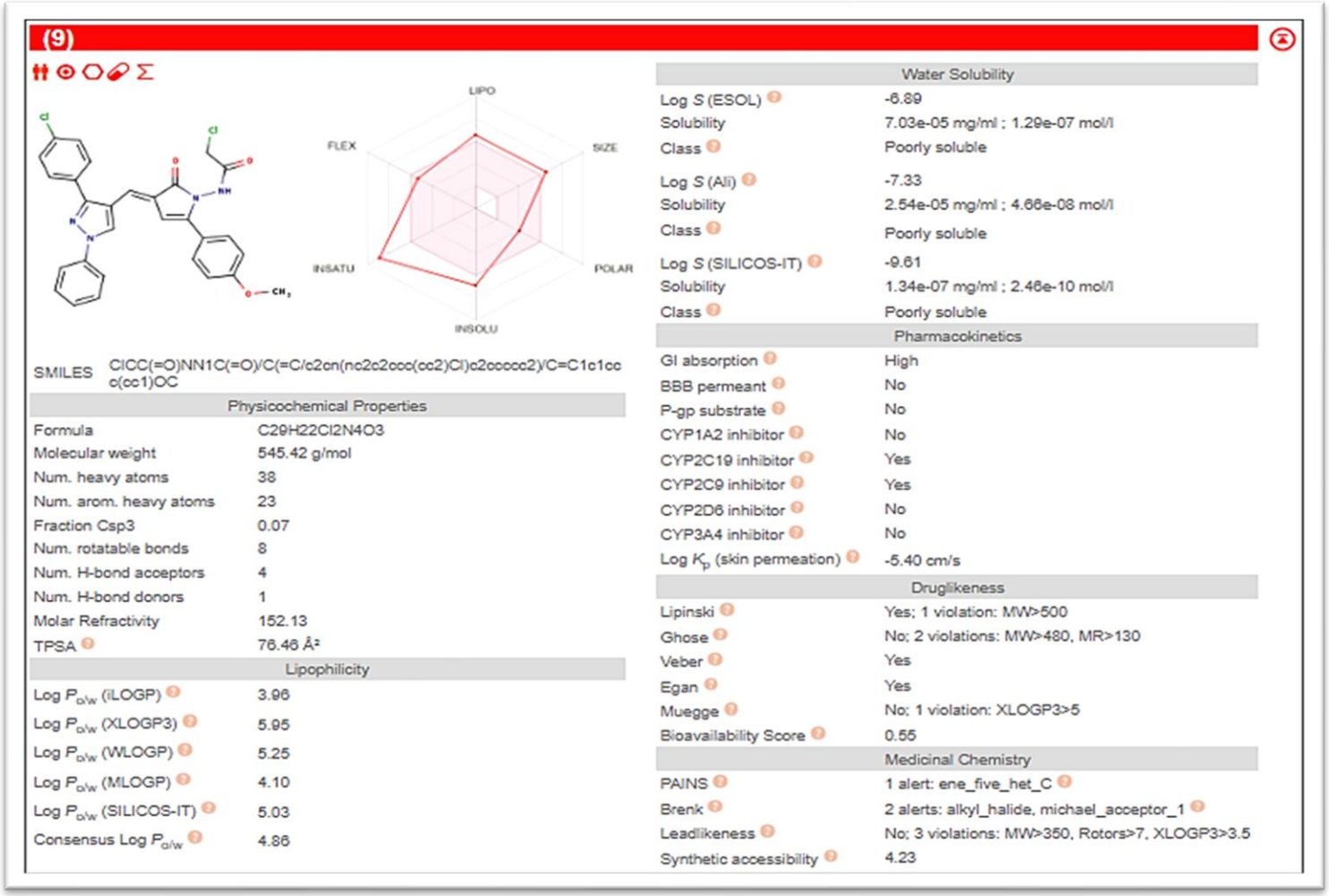


Fig. S8. ADME profile of compound 9.

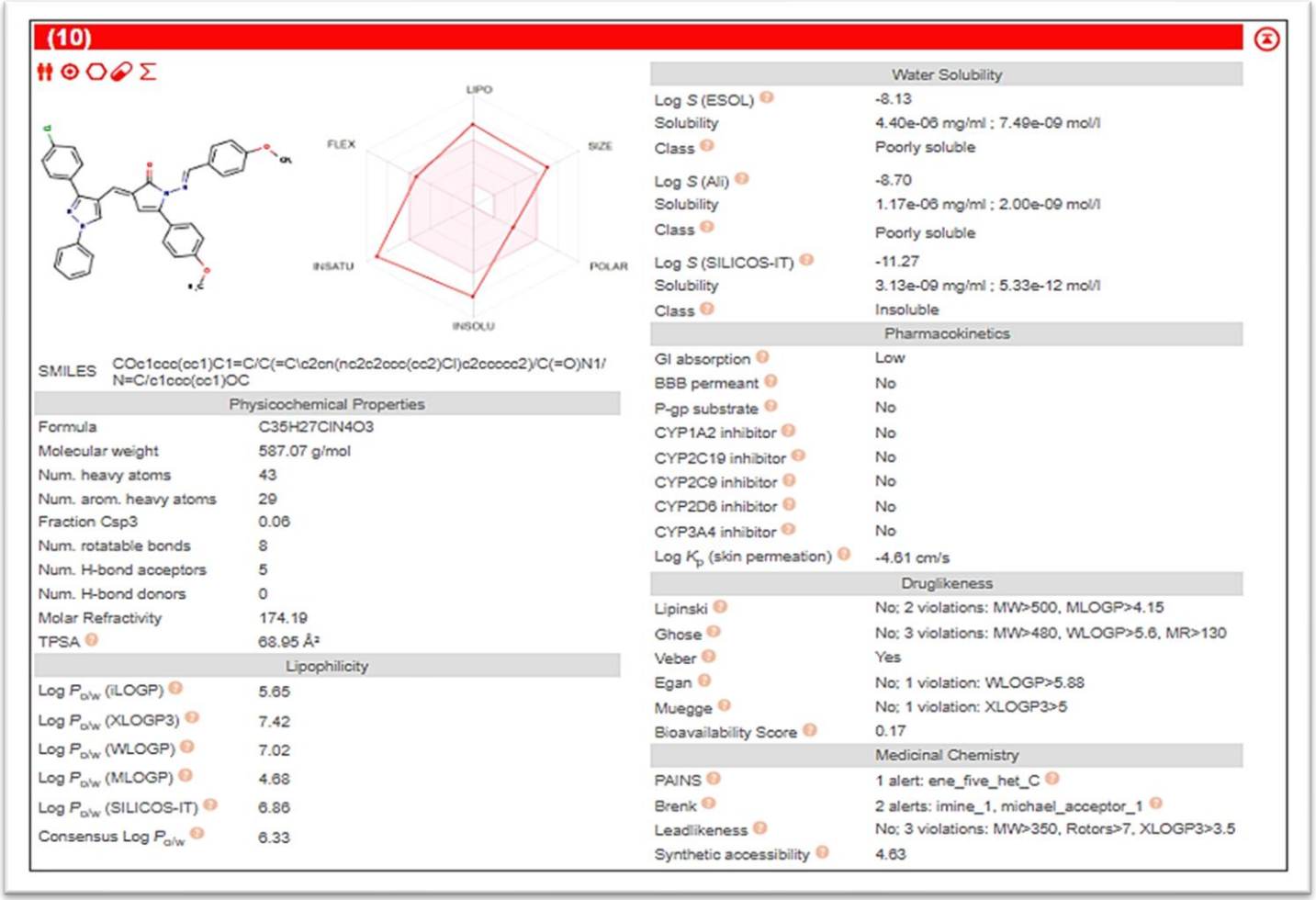


Fig. S9. ADME profile of compound 10.

Materials and methods

Antimicrobial activity

Microbial strains and general growth conditions

In the present study, five microbial strains were used to evaluate the antimicrobial activity of newly synthesized compounds: two Gram positive: *B. subtilis* (ATCC 6051) and *S. aureus* (ATCC 9144), two Gram negative: *E. coli* (ATCC 6633) and *P. aeruginosa* (ATCC 27853) and one fungal strain: *C. albicans* (ATCC 90028). Microbial standard strains were gifted from department of Microbiology and Immunology, Faculty of Pharmacy, Tanta University. Muller Hinton broth (MHB), Sabouraud dextrose agar (SDB) and broth (SDB) were generally used for bacterial and fungal growth respectively, at 37ºC for 24-30 h. The HDF cell lines were grown on Dulbecco's Modified Eagle medium (DMEM) that was supplemented with 10% fetal bovine serum and 0.1% antibiotic/antimycotic solution. All growth media and reagents used in this study were purchased form Sigma/Aldrich, USA, Oxoid, UK or Fluka, Switzerland.

Determination of Minimum Inhibitory Concentration (MIC)

The MICs of the evaluated compounds were determined using the standard broth dilution technique in accordance with CLSI guidelines [28]. Briefly, overnight cultures of reference microbial strains were diluted at a ratio of 1:1000 to achieve a final concentration of 1-2 × 10^5 CFU/mL. MICs were assessed through two-fold serial dilutions within a concentration range of 2 to 1000 µg/mL. Cultures were incubated at 37°C with constant shaking at 150 rpm, and microbial growth was evaluated by measuring the optical density at 600 nm. Each experiment was performed in triplicate.

Cytotoxicity testing

To investigate the cytotoxic effect of promising compounds, MTT on HDF cell viability have been employed. In summary, 0.5 × 10^5 cells/well in serum-free medium were plated in a flat bottom 96-well microplate and exposed to 20 µL of various doses 1-500 µg/mL of the tested compounds over 48 h at 37ºC in 5% CO_2. Following 4 h of incubation, the media were withdrawn, 40 µL of MTT solution/well was applied, and the absorbance at 570 nm was measured photometrically using microplate reader (Biotek Elx-808) [29].

Biofilm inhibition/eradication

Determination of Colony Count

Biofilms of *Pseudomonas aeruginosa* were formed in 96-well plates by inoculating each well with 100 µL of bacterial suspension (10^6 CFU/mL) and allowing cells to adhere for 1 h. The suspension was then removed, and wells were rinsed once with 100 µL PBS. Fresh growth medium (200 µL) was added, and plates were incubated at 37°C for 48 h to allow biofilm development. Following incubation, the medium was discarded and biofilms were gently washed with 100 µL PBS [30]. Treatments were applied using 100 µL PBS (control) or 0.5 of MIC concentrations of the tested compounds for 2 or 4 h. Biofilms were subsequently disrupted by pipetting, serially diluted, and plated on LB agar. Colony-forming units (CFU) were counted after incubation at 37°C for 18 h. All experiments were performed in triplicate [31].

Crystal violet (CV) assay

In this assay, *P. aeruginosa* at a concentration of 10^6 CFU/mL was cultured in 96-well microtiter plates with subinhibitory concentrations (Sub-MIC) of the tested formula (0.2-0.8 of the MIC value. After 24 h of incubation at 37°C, non-adherent (planktonic) bacteria were removed by rinsing in phosphate buffered saline (PBS, pH 7.2), and the attached bacterial biofilms were stained with crystal violet. Biofilm formation was then quantified using a microplate reader (Biotek Elx-808) at 590 nm, where the color intensity correlates with the degree of bacterial adhesion and biofilm development [31].

Potential antifungal mechanism

Sorbitol Protection Assay

The sorbitol protection assay was performed to evaluate whether the antifungal activity of the tested compounds involved disruption of the fungal cell wall. The assay was carried out using *C. albicans* (ATCC 90028) [32]. Briefly, two sets of broth microdilution assays were prepared following CLSI guidelines. In the first set, SDB medium without sorbitol was used as the standard control condition. In the second set, SBB medium was supplemented with 0.8 *M* sorbitol as an osmotic stabilizer. Two-fold serial dilutions of the tested compounds were prepared in 96-well microtiter plates (final volume 200 µL per well). Each well was inoculated with 100 µL of *C. albicans* suspension adjusted to 1×10^5 CFU/mL. Plates were incubated at

35°C for 48 h. The MIC was determined as the lowest concentration of the compound that showed no visible fungal growth.

A significant increase (≥2-fold) in MIC values in the presence of sorbitol compared with sorbitol-free medium was interpreted as evidence that the compound may target the fungal cell wall. All experiments were conducted in triplicate.

Ethidium bromide accumulation/efflux assay

The efflux pump inhibitory activity of tested compounds was assessed using EtBr accumulation and efflux assay. Briefly, bacterial cultures (*P. aeruginosa* PAO1) were grown to mid-logarithmic phase, washed, and resuspended in phosphate-buffered saline (PBS) to an OD600 of 0.2. For the accumulation assay, cells were incubated with EtBr (2 µg/mL) in the presence of test compounds at sub-inhibitory concentrations (0.25× MIC), vehicle control (1% DMSO), and positive control CCCP (75 µM), and fluorescence was monitored kinetically at 530 nm excitation/600 nm emission for 30 min using a microplate reader. For the efflux assay, cells were preloaded with EtBr (4 µg/mL) under energy-depleted conditions (with CCCP), washed, and resuspended in PBS containing 20 mM glucose with or without test compounds. The decrease in fluorescence was then recorded every minute for 20 min, and efflux rates were calculated from the fluorescence decay curves. Increased intracellular fluorescence in accumulation assays or reduced efflux rates compared with the vehicle control were interpreted as evidence of efflux pump inhibition [33].

Pharmacokinetics studies

The pharmacokinetic properties of newly synthesized derivatives and the reference drugs were evaluated using an *in-silico* approach *via* the SwissADME web tool (http://www.swissadme.ch/) [39-41]. This tool facilitated the comprehensive analysis of ADME profiles, alongside assessments of drug-likeness and other relevant physicochemical properties. Key parameters such as GI absorption, skin permeation (Log Kp), and interaction with cytochrome P450 isoenzymes were predicted to assess the compounds bioavailability, metabolic stability, and excretion patterns. Additionally, Lipinski rule of five was applied to determine the drug-likeness of the compounds, ensuring their potential suitability as orally active agents. The pharmacokinetic profiles were further visualized using the bioavailability radar and BOILED-Egg model, which predict passive GI absorption and brain penetration.

These predictions provided critical insights into the compound's potential effectiveness as antimicrobial agents, guiding the optimization of their pharmacokinetic properties for enhanced antimicrobial activity.