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Article

Usnic Acid Conjugates with Monoterpenoids as Potent Tyrosyl-DNA Phosphodiesterase 1 Inhibitors

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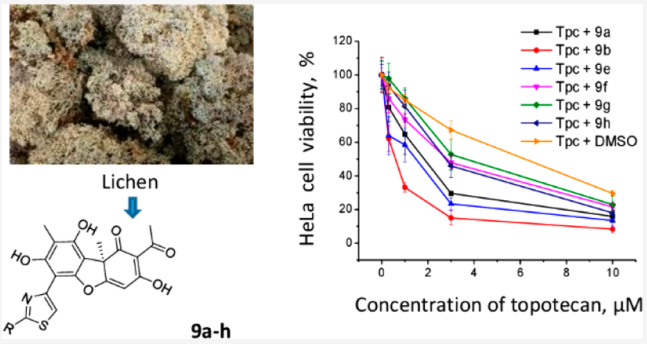
ABSTRACT: Hybrid molecules created from different pharmacophores of natural and synthetic equivalents are successfully used in pharmaceutical practice. One promising target for anticancer therapy is tyrosyl-DNA phosphodiesterase 1 (Tdp1) because it can repair DNA lesions caused by DNA-topoisomerase 1 (Top1) inhibitors, resulting in drug resistance. In this study, new hybrid compounds were synthesized by combining the pharmacophoric moiety of a set of natural compounds with inhibitory properties against Tdp1, particularly, phenolic usnic acid and a set of different monoterpenoid fragments. These fragments were connected through a hydrazinothiazole linker. The inhibitory properties of the new compounds mainly depended on the structure of the terpenoid moieties. The two most potent compounds, **9a** and **9b**, were synthesized from citral and citronellal, which contain acyclic fragments with IC_{50} values in the range of 10−16 nM. Some synthesized derivatives showed low cytotoxicity against HeLa cells and increased the effect of the Top1 inhibitor topotecan in vitro by three to seven times. These derivatives may be considered as potential agents for the development of anticancer therapies when combined with Top1 inhibitors.



The creation of hybrid molecules from natural compounds is a modern and a highly relevant trend in medicinal chemistry. This concept presents new perspectives regarding the use of natural compounds in pharmacology and can potentially improve the safety and efficacy of drugs.[1] A rational combination of ≥ two components of natural origin can lead to hybrid molecules that synergistically increase the efficacy of each other and consequently reduce the required dosage of the drug.[2] One approach regarding the concept of molecular hybridization is based on combining two different pharmacophores, with similar or different biological functions, directly or through a linker fragment.[3] This approach can resolve several common issues associated with the bioavailability of a drug, its transport through the membranes of cellular organelles, and protection against enzymatic degradation; consequently, it can improve its pharmacokinetic and pharmacodynamic profiles.[4] Additionally, the expected positive effects of hybrid molecules can include minimization of the risk of drug interaction and the development of resistance to therapy.[5,6] Several examples of hybrid molecules that exist in nature and form through various metabolic pathways (e.g., Taxol and cannabinoids) and are created from natural synthetic equivalents,[7] successfully used in pharmaceutical practice are present.

For several decades, usnic acid (UA) has attracted considerable research interest, due to the broad scope of its native biological activity, the susceptibility of its structure to chemical transformation, and its availability in high optical purity.[8,9] The primary obstacle to its widespread use in pharmacology is its high toxicity. Therefore, the successes achieved in the field of research and utilization of the biological properties of this compound during the past decades have been focused on the synthesis of its derivatives and the identification of structure−activity relationships.[10,11]

Recently, the use of UA (**1**)[12] and its derivatives **2**,[12−14] **3**,[12,15,16] and **4a** (Figure 1),[17] in the form of a combination therapy with cytotoxic drugs, has been widely studied using in vitro and in vivo experiments. This approach has been shown to result in pronounced effects that are associated with negating the tolerance of drug-resistant cancer cells[18] and the inhibition of tumor metastasis.[13,15] The mechanisms of action of these compounds have not been fully elucidated but include increasing the expression of E-cadherin[18] and inhibiting the growth of endovascular vessels,[19] as well as inhibiting DNA repair enzymes.[14,15,17]

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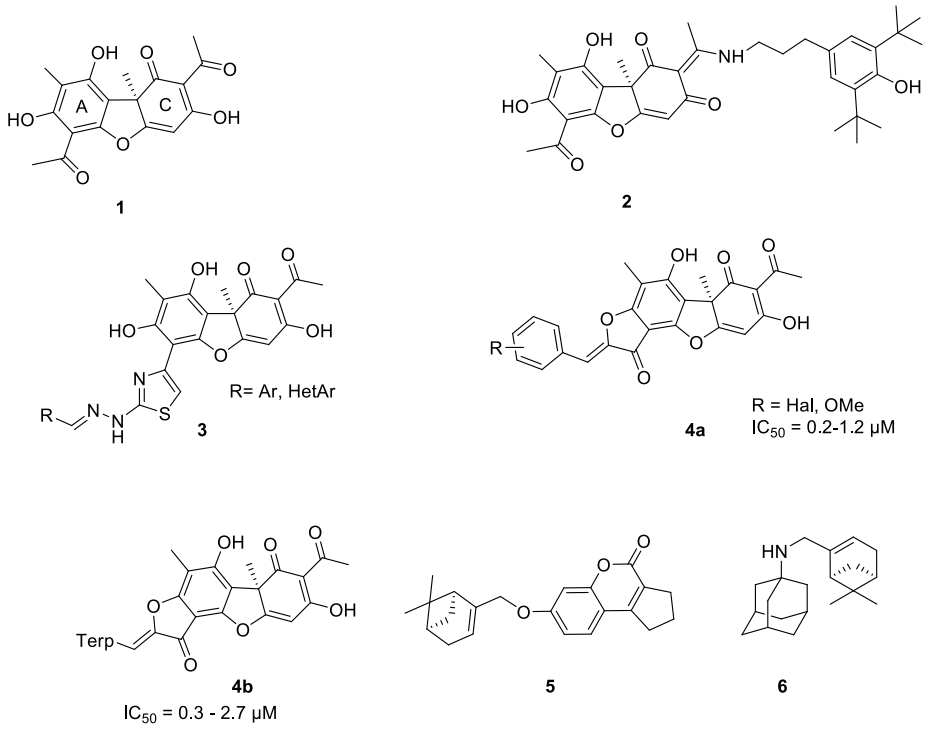

Figure 1. Natural and semisynthetic Tdp1 inhibitors.

The use of DNA repair enzyme inhibitors as chemotherapy components is a promising and an intensively developing area of modern oncopharmacology.[20] Using such compounds is particularly relevant for the treatment of drug-resistant tumors. One promising target for the treatment of oncological diseases involves the DNA repair enzyme tyrosyl-DNA phosphodiesterase 1 (Tdp1), which plays a key role in repairing damage to DNA caused by exposure to the camptothecin drugs, irinotecan and topotecan, which are widely used in clinical practice and are also involved in the repair of damage caused by drugs such as Temozolomide, bleomycin, and etoposide.[21] UA derivatives have proven to be promising inhibitors of repair enzymes. In particular, there are reports of their moderate inhibitory activity against PARP1[22] and significantly more pronounced activity (IC_{50} 10^{-6}–10^{-8} M) against Tdp1,[14−17,23,24] whereas native UA (**1**) does not demonstrate such an effect. Recent studies have revealed certain structural patterns of potential inhibitors based on UA, leading to increased activity. In particular, it was found that thiazole derivatives **3** of UA, with arylhydrazone substituents attached to the thiazole ring, exhibit Tdp1 inhibitory activity in the nanomolar range.[15,16] In vitro studies with lung carcinoma cell line A-549[16] and in vivo studies in mice with Lewis lung carcinoma[15] have confirmed the synergistic antitumor effect of thiazole derivatives of UA when combined with topotecan. Studies on the purified enzyme have also demonstrated that the structure of the aryl or heteroaryl substituent significantly affected the inhibitory potential of compounds.[15,16]

This study aimed at the synthesis and study of the Tdp1 inhibitory activity of the hydrazinothiazole derivatives of UA containing terpenoid substituents of natural origin. The choice of monoterpenoids as the second component of the inhibitor molecule was based on the literature data related to the activity of Tdp1 inhibitors combining monoterpenoid moieties with substituted 7-hydroxycoumarin (**5**),[25] adamantane fragments (**6**), an amino linker,[26] amide,[27] or ester bond,[28] or an aromatic/heteroaromatic moiety.[29] These compounds showed inhibitory activity in the 0.6−21 μM range and demonstrated a synergistic effect in vitro in human cell lines when used in combination with a Top1 inhibitor, camptothecin (coumarins)[25] or topotecan (adamantanes).[26,27] The advantages of terpenoids as natural compounds include their structural affinity to many biological receptors developed during the course of evolution and their own wide range of biological activities, which have often inspired the development of synthetic drugs based on them.[30] The introduction of terpenoid fragments—which often have a bulky chiral 3D structure—onto the Tdp1 inhibitor molecule may also contribute to more selective binding of the ligand to the enzyme. It has been demonstrated that the use of the available and cheap monoterpenoids as starting molecules for synthesizing new and effective agents to treat a number of diseases is a rather promising and topical direction of medicinal chemistry. It should also be mentioned that the use of monoterpenoids to design drugs often reduces toxicity of the resulting compound, which may indicate that there is complementarity between natural molecules and living organisms that have coexisted for thousands of years.

The spectrum of UA derivatives described in the literature is fairly broad; however, UA derivatives that combine the structure of phenols and terpenoids are represented only by alkylidenfuranone derivatives **4b**.[24] These compounds display Tdp1 inhibitory properties that are not inferior to the analogues featuring a benzylidenefuranone fragment **4a**.[17]

Herein, we synthesized new hybrid derivatives of terpenophenol, i.e., UA and monoterpenoids that are joined into a single molecule by a hydrazinothiazole linker. The synthesized compounds include substances containing both cyclic (monocyclic and bicyclic) and acyclic terpene fragments. A compound with a non-terpene but saturated linear substituent was synthesized to enable a broader investigation of the structure−

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activity relationship. Studies of Tdp1 inhibitory activity and cytotoxicity, concerning transplantable cell lines, were conducted for the series of new UA derivatives.

RESULTS AND DISCUSSION

Chemistry. Terpenoid thiosemicarbazones **7a−h** were synthesized from commercially available terpenoid aldehydes and ketones (Figure 2; Scheme 1), according to published

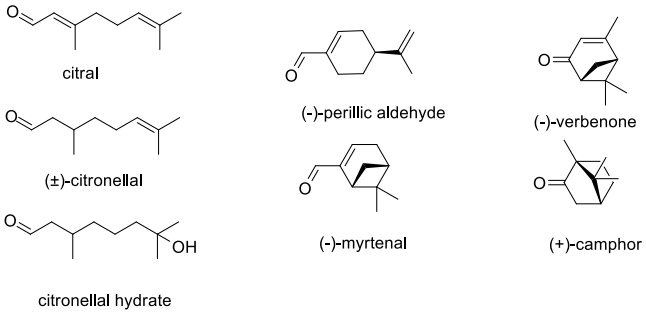

Figure 2. Structures of monoterpenoids used for synthesis.

methods or under conditions similar to those described. In all cases, except for verbenone thiosemicarbazone, only one set of signals, corresponding to one isomer of thiosemicarbazone, was observed in the NMR spectra. In the case of verbenone (**7g**), two sets of peaks—for the *E* and *Z* isomers—were observed in a 3:2 ratio. Citronellal and citronellal hydrates were used as racemates, and the resulting derivatives, **9b** and **9c**, were present as a mixture of two diastereomers; no doubling of signals in the NMR spectra was observed. The compounds were further tested as a mixture of diastereomers.

UA bromoketone **8** was obtained from *R*-(+)-UA **1** by the action of bromine in dioxane, in the presence of hydrogen bromide, according to the method described by Luzina et al.[31] The synthesis of target hydrazinothiazole derivatives **9a−h** was conducted by the treatment of bromo-UA **8** with thiosemicarbazones **7a−h** (Scheme 1).[32] The reaction was conducted in MeOH at reflux for 1 h, and the final products, isolated by column chromatography, with yields, are reported in Table 1. Column chromatography was used to purify the final products. Compound **9f** was obtained according to the procedure provided above.[32] The structures of all new compounds were confirmed by ^{1}H and ^{13}C NMR and HRMS data. The regioselectivity of bromination and further cyclization to thiazole derivatives were proven early for analogues of compounds **9a−h**, which were characterized on the basis of X-ray structural analysis (RSA).[33]

Biology. A fluorophore/quencher-coupled DNA biosensor for real-time measurement of Tdp1 cleavage activity was used. The substrate was a 16-mer single-strand oligonucleotide containing both a 5′-FAM fluorophore donor and a quenching 3′-BHQ1 moiety. The IC_{50} values (half-maximal inhibition concentrations) for compounds **9a−h** are collated in Table 1.

High Tdp1 inhibitory activity (in the nanomolar concentration range) was established for terpenic derivatives of UA with a hydrazinothiazole linker **9**. Apparent relationships were observed between the effective inhibitory concentrations of the compounds and the structure of the terpene fragment; conjugates of (+)-UA with acyclic fragments **9a** and **9b** were among the compounds with high inhibitory ability. The compound with monocyclic substituent **9e** was marginally less active. Derivatives with bicyclic substituents **9f−h** had three to five times higher IC_{50} values. Remarkably, both the replacement of the isoprene fragment with a saturated linear fragment (**9d**) and the introduction of a polar hydroxy group (**9c**) led to a significant decrease in activity; the inhibitory properties of the conjugates decreased by an order of magnitude in both cases.

Cytotoxicity. Because Tdp1 inhibitors are supposed to be used in therapeutic cocktails, they should possess the lowest possible toxicity and should not enhance the existing side effects of therapy. Intrinsic cytotoxicity was studied for substances that showed anti-Tdp1 activity in concentrations of <80 nM. The

Scheme 1. Synthesis of Hydrazonothiazole Derivatives 9a−h

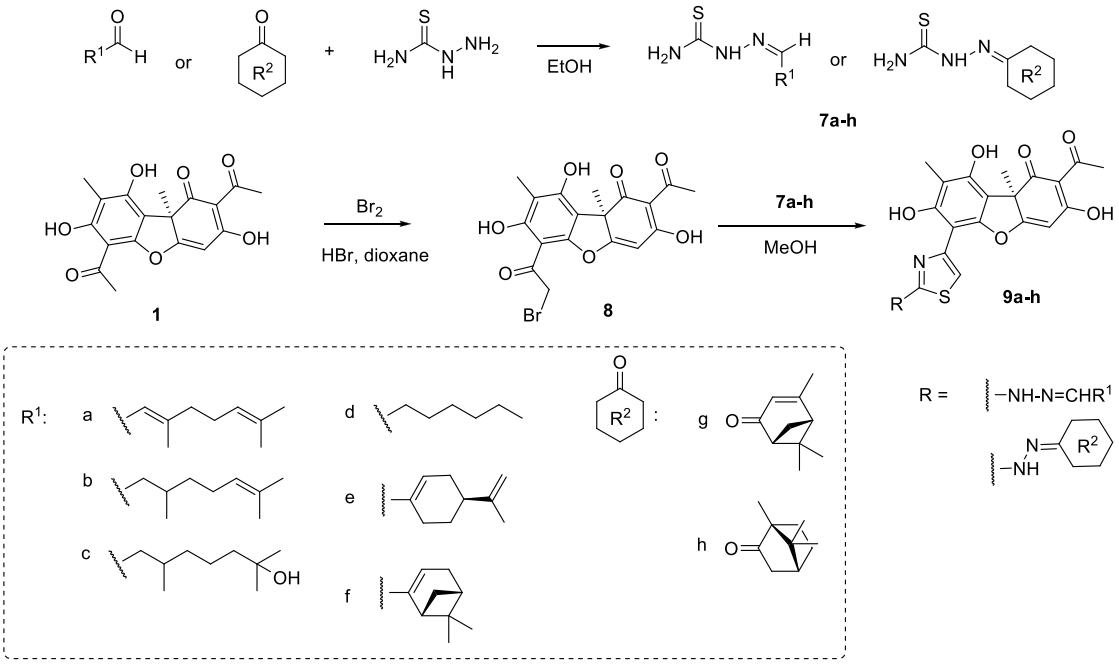

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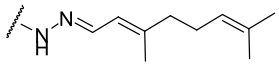
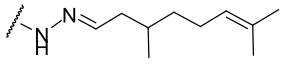
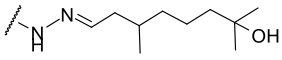
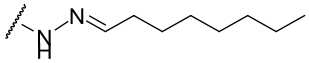
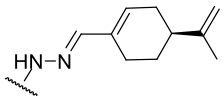
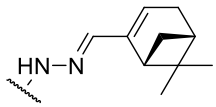
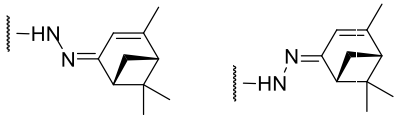
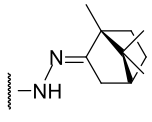
Table 1. Influence of UA Derivatives on Tdp1 Activity[a]

Compound	R =	Yield %	IC_{50} (nM)	CC_{50} for Tpc (μM)*	Enhancement of cytotoxity of Tpc
Tpc				4.5	
9a		64	10.3±0.4	1.5	3.0
9b		44	16.4±0.1	0.63	7.1
9c		63	139±1	nd**	nd
9d		82	88±16	nd	nd
9e		73	27±2	1.2	3.8
9f		66	45±12	2.7	1.7
9g		62	46±2	3.5	1.3
9h		91	31±11	2.7	1.7

[a]*Topotecan (Tpc) in the presence of 5 μM Tdp1 inhibitors. **nd = not determined.

cytotoxicity of compounds was investigated using HeLa (cervical cancer) cells (Figure 3).

UA conjugates with terpenoids had a moderate cytotoxic effect in concentrations from 20 μM, which exceeded inhibitory concentrations by 2 to 3 orders of magnitude. Compound **9f**, synthesized from myrtenal, was the least toxic for both cell types (CC_{50} > 100 μM), and for the remaining compounds, the values of CC_{50} were in the 20−70 μM range. Notably, compounds based on myrtenal were reported as the most promising in the series of terpenoid derivatives with coumarins and adamantanes,

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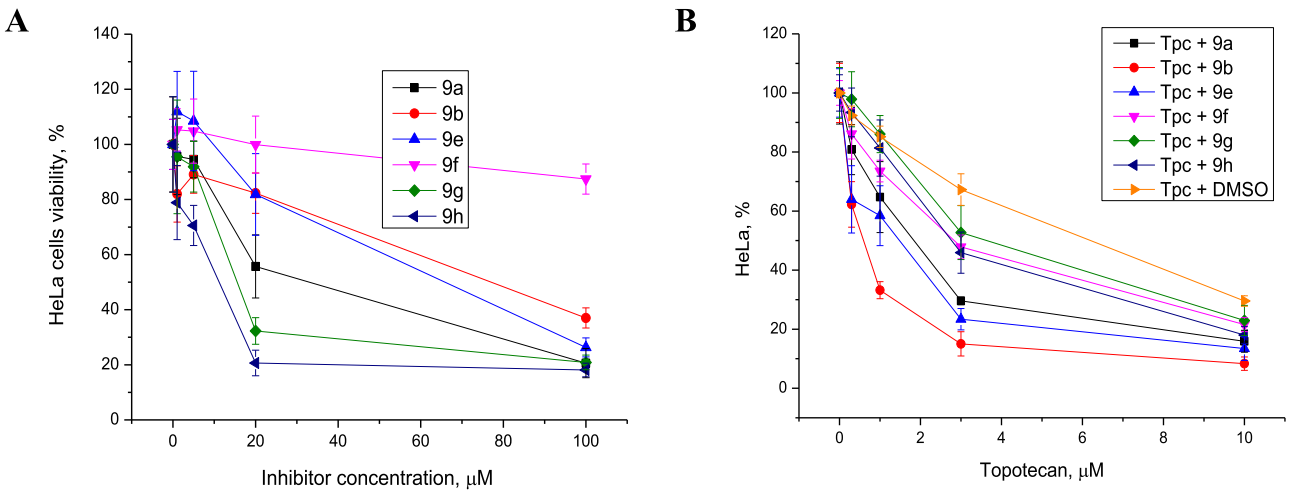

Figure 3. (A) Dose-dependent impact of UA derivatives on the viability of HeLa cells. (B) Dose-dependent action of topotecan in combination with UA derivatives on HeLa cells' viability.

previously synthesized as Tdp1 inhibitors, which may indicate the important role of this structural unit for the development of potential Tdp1 inhibitors.[25,26]

Topoisomerase 1 (Top1) poisons (topotecan and irinotecan) are used as anticancer drugs for the treatment of a wide range of oncological diseases.[34,35] Because Tdp1 is involved in the removal of DNA damage caused by Top1 poisons, it is believed to be responsible for the drug resistance of some cancers.[36] Thus, a combination of Top1 poisons and Tdp1 inhibitors may be able to improve the effectiveness of chemotherapy.

Next, the effect of Tdp1 inhibitors on the cytotoxic effect of topotecan, a Top1 poison used in clinical practice, was investigated. Nontoxic concentrations of UA derivatives (5 μM) and different concentrations of topotecan were used to estimate CC_{50} values for HeLa cells. Tdp1 inhibitors in nontoxic concentrations increased the cytotoxic effect of topotecan from 1.7 to 7 times (Figure 3B and Table 1). The best results were obtained for compound **9b**, which reduced the CC_{50} of topotecan 7-fold.

Thus, terpenophenol UA derivatives represent a promising class of compounds for development as sensitizers of Top1 poisons to achieve enhanced therapeutic effects.

Molecular Modeling. Molecules **9b** and **9c** were docked into the binding site of Tdp1 (PDB ID: 6N19, resolution 1.5 Å) to clarify the 10-fold difference in the values of their effective inhibitory concentrations. The structures of the complexes of the catalytic domain of Tdp1 with compounds **9b** and **9c** with the lowest energy were used to analyze contacts in the complex. An analysis of the contacts of compound **9b** with the active center showed that the O5 oxygen atom of this compound formed a hydrogen bond with the catalytic residue His 263, along with Lys 265 and Asn 283, whereas the sulfur atom formed hydrogen bonds with Pro 461 and Thr 466 (Figure 4A). The energy of the hydrogen bonds of compound **9b** with catalytic domain Tdp1 was −10.0 kcal/mol.

The O6 of the hydroxy group of the aliphatic fragment **9c** formed hydrogen bonds with the catalytic residues His 263 and Lys 265, and O5 formed a hydrogen bond with Lys 519, alongside Tdp1 (Figure 4B). The Tyr 204 residue of the Tdp1 formed an arene−H bond with compound **9c**. The energy of hydrogen bonds of compound **9c** alongside the catalytic domain of Tdp1 was −4.2 kcal/mol.

Thus, the binding of the hydroxy group of the aliphatic fragment of inhibitor molecule **9c** to the active site of the enzyme led to the loss of contact of the dibenzofuran part of the inhibitor molecule with Tdp1 catalytic residues, which in turn gave rise to a decrease in total binding energy. Inhibitors **9b** and **9c** had the same binding site to the catalytic domain (Figure 5). The binding energies of compounds **9b** and **9c** with the enzyme correlated with the Tdp1-inhibiting activity of compound **9b** at a lower concentration, compared with compound **9c** (IC_{50} values of 16.4 and 139 nM).

In conclusion, new compounds in the form of conjugates of UA and monoterpenoids were developed that can serve as effective inhibitors of Tdp1, in the concentration range of 10−139 nM. These compounds serve as one of the most effective inhibitors of Tdp1 published to date. Summarizing the data on the inhibitory effect and toxicity, compound **9b** can be considered as the most promising inhibitor of Tdp1 because it was weakly toxic to the studied cells, demonstrated an inhibitory effect on Tdp1 at a concentration of 16 nM, and enhanced the cytotoxicity of topotecan 7-fold.

EXPERIMENTAL SECTION

Chemistry. The ^{1}H and ^{13}C NMR spectra for solutions of the compounds in $CDCl_3$ were recorded using a Bruker AV-400 spectrometer (400.13 and 100.61 MHz, respectively). The residual signals of the solvent were used as references (δ_H 2.48 and δ_C 39.52 for DMSO-d_6 and δ_H 7.27 and δ_C 77.1 for $CDCl_3$). The mass spectra (70 eV) were recorded on a DFS Thermo Scientific high-resolution mass spectrometer. Electron impact ionization was used in the measurement of mass spectra. Merck silica gel (63−200 μm) was used for column chromatography. Thin-layer chromatography was performed on Silufol plates (UV-254).

(+)-UA **1** ($[\alpha]_D$ +478 (*c* 0.1, $CHCl_3$)) was isolated from a mixture of lichens of the genus *Usnea*, using the procedure employed in Salakhutdinov et al.[37]

The atom numbers in the compound were provided for the assignment of signals in the NMR spectra and were different from numbering in the nomenclature name. The target compounds (**9a−h**) had a purity of at least 97% (high-performance liquid chromatography).

General Procedure for the Synthesis of Compounds 7a−h. Aldehyde (1 mmol) and thiosemicarbazide (1 mmol) were added to a flask. The mixture was dissolved in 10 mL of EtOH (95%). The mixture was boiled, and after 60 min, it was cooled, and water was added. The precipitate was filtered off, washed with water, and then air-dried. The thiosemicarbazones **7a−h** obtained were isolated in 81−98% yields.

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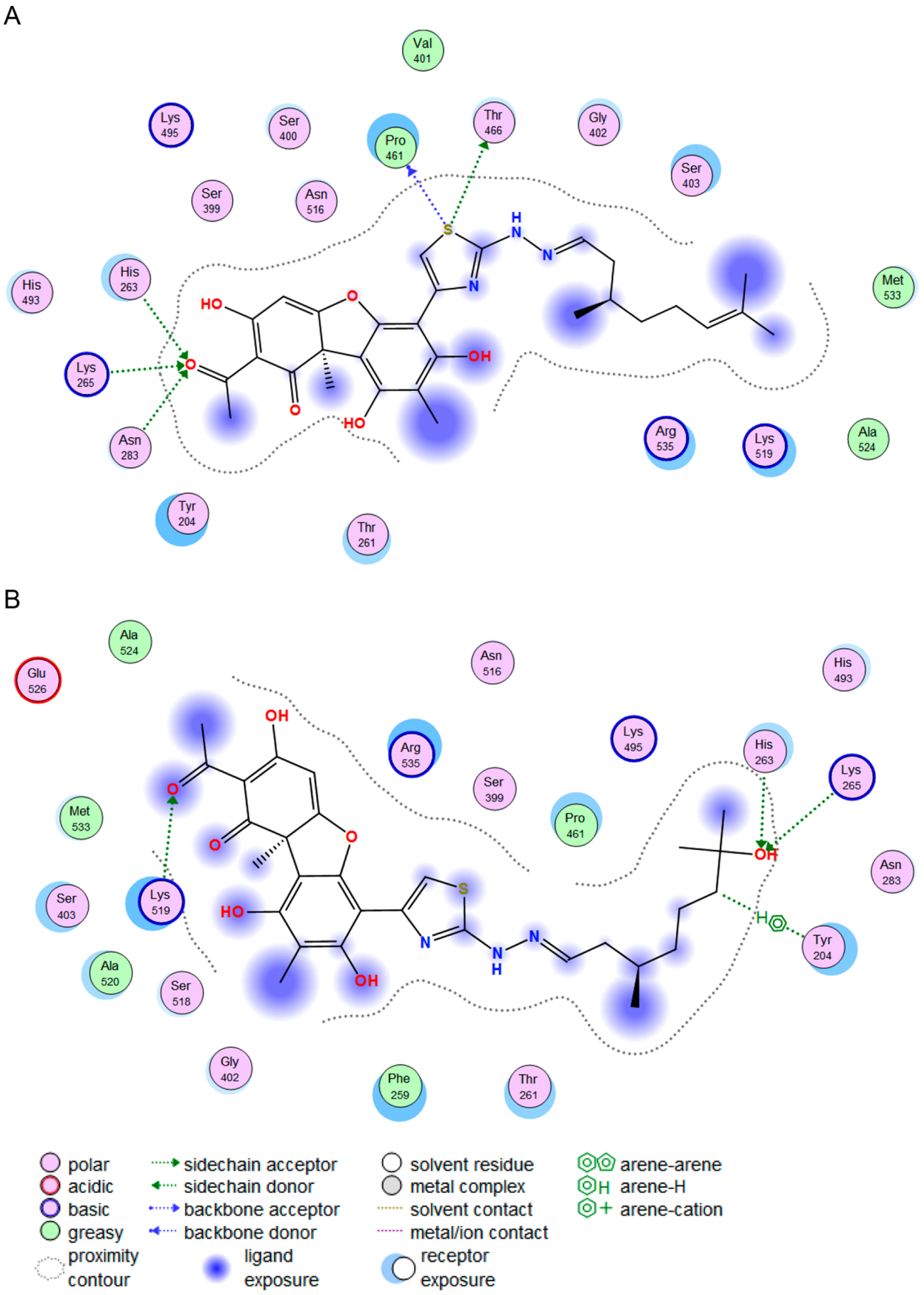

Figure 4. 2D diagrams of the contacts of compounds **9b** (A) and **9c** (B) with catalytic domain Tdp1, obtained using the "ligand interaction" module of the MOE 2019 software package. Pin designations are indicated in the figure.

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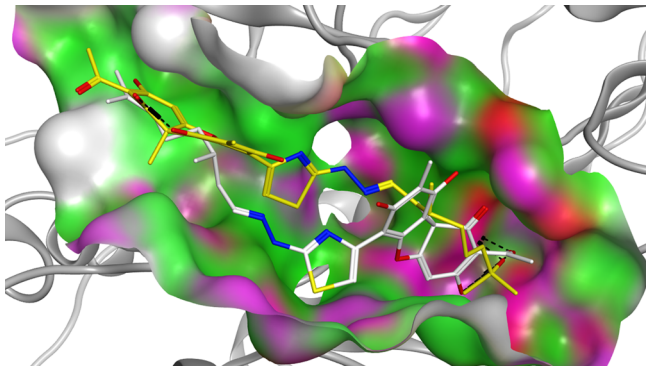

Figure 5. Superposition of compounds **9b** and **9c** in the active center of the catalytic domain of Tdp1. The structures of the compounds are shown in stick form in white (**9b**) and yellow (**9c**). The molecular surface of Tdp1 at the binding site is shown as hydrophobic (green), polar (pink), and exposed (red).

Compounds **7a** and **7b** were assigned as described in Tarasconi et al.[38] Compound **7d** was assigned as described in Wiles et al.[39] Compound **7f** was assigned as described by Oliveira et al.[40] Compound **7h** was assigned as described by Lazkowski et al.[41]

*(R,E)-2-{[4-(Prop-1-en-2-yl)cyclohex-1-enyl]methylene}-hydrazinecarbothioamide (**7e**).* White crystals, yield 81%; mp 138−140 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.46 (1H, m), 1.72 (3H, s), 1.85−2.46 (6H, m), 4.69 (2H, d, J = 18.8 Hz), 6.17 (1H, s), 6.51 (1H, s), 7.05 (1H, s), 7.55 (1H, s), 10.02 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.5, 23.3, 26.5, 31.4, 40.6, 109.0, 134.0, 138.4, 147.2, 148.6, 177.8.

*{[(1S,2E,5S)-4,6,6-Trimethylbicyclo[3.1.1]hept-3-en-2-ylidene]-amino}thiourea and {[(1S,2Z,5S)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ylidene]amino}thiourea (3:2) (**7g**).* White, amorphous powder, yield 98%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.81 (3H, s), 1.42 (3H, s), 1.49 (1H, m), 1.87 (3H, s), 2.23 (1H, m), 2.65 (2H, m), 3.41 (1H, m), 6.68 (1H, s), 7.50 (1H, s), 7.93 (1H, s), 10.36 (1H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 21.7, 23.6, 25.9, 37.3, 47.9, 48.6, 50.7, 110.7, 153.9, 160.3, 177.9; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.81 (3H, s), 1.39 (3H, s), 1.58 (1H, m), 1.90 (3H, s), 2.29 (1H, m), 2.62 (2H, m), 3.53 (1H, m), 5.77 (1H, s), 7.50 (1H, s), 7.93 (1H, s), 10.27 (1H, s); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 21.9, 22.9, 25.5, 36.5, 43.1, 47.7, 48.4, 118.5, 155.7, 156.7, 178.1.

General Procedure for the Synthesis of Compounds 9a−h. (+)-UA **1** (1 mM) was treated with the bromine−dioxane complex (2 mmol Br_2, 0.10 mL, dissolved in dioxane, 14 mL) and several drops of HBr and left for 7 d at room temperature. The reaction mixture was concentrated in a rotary evaporator and chromatographed over silica gel with CH_2Cl_2. The yield of compound **8** was 67%.

A 1 mmol amount of compound **8** was added to 1 mmol of the thiosemicarbazone of aldehyde **7a−h**, and the mixture was boiled in 10 mL of MeOH for 1 h. After 60 min the mixture was cooled, and water was added to the mixture. The precipitate that formed was filtered off, washed with water, and air-dried.

The NMR assignment is in the Supporting Information (Tables S1−S3).

*(2R)-4-Acetyl-10-{2-[(E)-2-[(2E)-3,7-dimethylocta-2,6-dien-1-ylidene]hydrazin-1-yl]-1,3-thiazol-4-yl}-5,11,13-trihydroxy-2,12-dimethyl-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),4,6,10,12-entaen-3-one (**9a**).* Yellow, amorphous powder, yield 64%; mp 145−150 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.59 (3H, s), 1.66 (3H, s), 1.71 (3H, s), 1.84 (3H, s), 2.14 (7H, s), 2.63 (3H, s), 5.09 (1H, bs), 5.89 (1H, s), 6.00 (1H, d, J = 9.1 Hz), 7.08, (1H, s), 7.70 (1H, d, J = 9.1 Hz), 9.09 (1H, bs), 10.25 (1H, bs), 18.78 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.0, 16.7, 17.3, 25.2, 25.7, 27.4, 31.7, 39.7, 59.1, 97.0, 97.3, 102.9, 104.0, 104.8, 108.5, 120.4, 122.6, 131.8, 142.2, 143.1, 147.6, 150.9, 151.2, 156.1, 165.9, 180.4, 191.3, 197.8, 201.0; HRMS m/z 549.1925 (calcd for $C_{29}H_{31}O_6N_3S$, 549.1928).

*(2R)-4-Acetyl-10-{2-[(E)-2-(3,7-dimethyloct-6-en-1-ylidene)-hydrazin-1-yl]-1,3-thiazol-4-yl}-5,11,13-trihydroxy-2,12-dimethyl-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),4,6,10,12-pentaen-3-one (**9b**).* Yellow, amorphous powder, yield 44%; mp 120−124 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.94 (3H, d, J = 6.3 Hz), 1.10−1.28 (3H, m),1.34−1.42 (1H, m), 1.59 (3H, s), 1.67 (3H, s), 1.73 (3H, s), 1.90−2.05 (2H, m), 2.13−2.22 (4H, m), 2.63 (3H, s), 5.08 (1H, t, J = 7.1 Hz), 5.90 (1H, s), 7.09 (1H, s), 7.21 (1H, t, J = 5.7 Hz), 8.64 and 8.80 (1H, bs), 10.24 (1H, s), 13.29 (1H, bs), 18.77 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 17.6, 19.5, 25.3, 25.6, 27.8, 30.9, 32.1, 36.6, 39.0, 59.4, 97.3, 97.4, 103.2, 104.2, 105.1, 108.9, 124.1, 131.5, 145.6, 146.1, 151.2, 151.5, 156.5, 166.6, 180.7, 191.6, 198.1, 201.3; HRMS m/z 551.2082 (calcd for $C_{29}H_{33}O_6N_3S$, 551.2085).

*(2R)-4-Acetyl-5,11,13-trihydroxy-10-{2-[(E)-2-(7-hydroxy-3,7-dimethyloctylidene)hydrazin-1-yl]-1,3-thiazol-4-yl}-2,12-dimethyl-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),4,6,10,12-pentaen-3-one (**9c**).* Yellow, amorphous powder, yield 63%; mp 135−139 °C; ^{1}H NMR ($CDCl_3$, 400 Hz) δ 0.94 (3H, d, J = 6.7 Hz), 1.18−1.22 (7H, m), 1.34−1.46 (7H, m), 1.72 (3H, s), 2.13 (3H, s), 2.16−2.19 (1H, m), 2.64 (3H, s), 5.89 (1H, s), 7.08 (1H, s), 7.17 (1H, t, J = 5.6 Hz), 8.73 (1H, bs), 10.24 (1H, s), 12.37 (1H, bs), 18.77 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.3, 19.6, 21.5, 27.8, 29.2, 29.2, 31.3, 32.1, 36.9, 39.1, 43.9, 59.5, 70.9, 97.3, 97.5, 103.2, 104.2, 105.1, 108.9, 143.5, 146.3, 151.2, 151.5, 156.6, 166.6, 180.7, 191.6, 198.1, 201.3; HRMS m/z 569.2187 (calcd for $C_{29}H_{35}O_7N_3S$, 569.2190).

*(2R)-4-Acetyl-5,11,13-trihydroxy-2,12-dimethyl-10-{2-[(E)-2-octylidenehydrazin-1-yl]-1,3-thiazol-4-yl}-8-oxatricyclo[7.4.0.0^{2,7}]-trideca-1(9),4,6,10,12-pentaen-3-one (**9d**).* Yellow, amorphous powder, yield 82%; mp 100−105 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86−0.89 (3H, m), 1.20−1.35 (8H, m), 1.54 (2H, bt), 1.73 (3H, s), 2.13 (3H, s), 2.31 (2H, bt), 2.64 (3H, s), 5.91 (1H, s), 7.09 (1H, s), 7.18 (1H, bt), 8.53 (1H, bs), 10.23 (1H, s), 18.77 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 13.9, 22.4, 26.3, 27.7, 28.8, 28.9, 31.5, 31.9, 32.0, 59.3, 97.2, 97.4, 103.1, 104.9, 105.0, 108.8, 143.3, 147.1, 151.1, 151.4, 156.5, 166.5, 180.7, 191.5, 198.0, 201.3; HRMS m/z 525.1927 (calcd for $C_{27}H_{31}O_6N_3S$, 525.1928).

*(2R)-4-Acetyl-5,11,13-trihydroxy-2,12-dimethyl-10-{2-[(E)-2-{[(4R)-4-(prop-1-en-2-yl)cyclohex-1-en-1-yl]methylidene}hydrazin-1-yl]-1,3-thiazol-4-yl}-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),4,6,10,12-pentaen-3-one (**9e**).* Yellow, amorphous powder, yield 73%; mp 143−145 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50 (1H, bs), 1.75 (3H, s), 1.76 (3H, s), 1.92 (1H, bs), 2.12−2.26 (5H, m), 2.28−2.36 (1H, m), 2.52−2.62 (1H, m), 2.67 (3H, s), 4.76 (2H, d, J = 18.5 Hz), 5.93 (1H, s), 6.01 (1H, s), 7.11 (1H, s), 7.34 (1H, s), 8.72 (1H, s), 10.28 (1H, s), 12.58 (1H, s), 18.81 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 20.6, 23.4, 26.6, 27.7, 31.2, 32.1, 40.7, 59.4, 97.2, 97.5, 103.2, 104.3, 105.1, 108.8, 108.9, 134.3, 135.2, 143.4, 146.1, 148.8, 151.1, 151.4, 156.4, 166.5, 180.6, 191.5, 198.0, 201.2; HRMS m/z 547.1767 (calcd for $C_{29}H_{29}O_6N_3S$, 547.1772).

*(2R)-4-Acetyl-10-{2-[(E)-2-{[(1R,5S)-6,6-dimethylbicyclo[3.1.1]-hept-2-en-3-yl]methylidene}hydrazin-1-yl]-1,3-thiazol-4-yl}-5,11,13-trihydroxy-2,12-dimethyl-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1-(9),4,6,10,12-pentaen-3-one (**9f**).* Yellow, amorphous powder, yield 66%; mp 155−160 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.78 (3H, s), 1.35 (3H, s), 1.75 (3H, s), 2.17 (5H, bs), 2.38−2.60 (3H, m), 2.67 (3H, s), 2.94 (1H, t, J = 5.6 Hz,), 5.88 (1H, m), 5.91 (1H, s), 7.07 (1H, s), 7.43 (1H, s), 9.05 (1H, bs), 10.27 (1H, s), 18.77 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 20.7, 25.9, 27.7, 30.9, 32.0, 32.2, 37.5, 40.0, 40.6, 59.3, 97.2, 97.5, 103.2, 104.3, 105.1, 108.9, 131.7, 143.2, 144.7, 144.7, 151.2, 151.5, 156.4, 166.5, 180.6, 191.5, 198.1, 201.2; HRMS m/z 547.1728 (calcd for $C_{29}H_{29}O_6N_3S$, 547.1772).

*(2R)-4-Acetyl-5,11,13-trihydroxy-2,12-dimethyl-10-(2-{2-[(2Z)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ylidene]hydrazin-1-yl}-1,3-thiazol-4-yl)-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),4,6,10,12-pentaen-3-one and (2R)-4-acetyl-5,11,13-trihydroxy-2,12-dimethyl-10-(2-{2-[(2E)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ylidene]-hydrazin-1-yl}-1,3-thiazol-4-yl)-8- oxatricyclo[7.4.0.0^{2,7}]trideca-1-(9),4,6,10,12-pentaen-3-one (3:2) (**9g**).* Yellow, amorphous powder, yield 62%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (3H, s), 1.44 (3H, s), 1.46−1.54 (1H, m), 1.72 (3H,s), 1.92 (3H, s), 2.12 (3H, s), 2.39 (1H, bt), 2.55−2.73 (5H, m), 2.79 (2H, m), 5.89 (1H, s), 6.09 (1H, s), 7.09 (1H, s), 8.58 (1H, bs), 10.22 (1H, s), 12.37 (1H, bs), 18.79 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 21.2, 23.9, 25.9, 27.7, 32.0, 37.6, 49.5,

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50.9, 57.4, 59.4, 97.2, 97.6, 103.0, 104.2, 105.1, 108.1, 108.9, 143.5, 143.6, 151.1, 151.5, 154.1, 156.3, 156.7, 166.5, 167.4, 180.8, 191.6, 198.1, 201.2. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.91 (3H, s), 1.45 (3H, s), 1.48 (1H, m), 1.72 (3H,s), 1.92 (3H, s), 2.12 (3H, s), 2.32 (1H, bt), 2.55−2.73 (5H, m), 5.89 (1H, s), 5.93 (1H, s), 7.08 (1H, s), 8.45 (1H, bs), 10.22 (1H, s), 12.37 (1H, bs), 18.79 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 22.1, 23.4, 26.5, 27.8, 32.0, 36.7, 40.7, 49.6, 57.5, 59.5, 97.2, 97.6, 103.0, 104.2, 105.1, 108.9, 118.6, 143.5, 143.6, 151.1, 151.5, 155.0, 156.3, 161.9, 166.5, 167.6, 180.8, 191.6, 198.1, 201.2; HRMS *m*/*z* 547.1777 (calcd for $C_{29}H_{29}O_6N_3S$, 547.1772).

*(2R)-4-Acetyl-5,11,13-trihydroxy-2,12-dimethyl-10-(2-{2-[(2Z)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ylidene]hydrazin-1-yl}-1,3-thiazol-4-yl)-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),4,6,10,12-pentaen-3-one (**9h**).* Yellow, amorphous powder, yield 91%; mp 176−178 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.77 (3H, s), 0.94 (3H, s), 1.05 (3H, s), 1.17−1.29 (1H, m), 1.39−1.49 (1H, m), 1.71 (3H, s), 1.74−1.80 (1H, m), 1.82−1.92 (2H, m), 2.00−2.08 (1H, m), 2.13 (3H, s), 2.36−2.46 (1H, m), 2.63 (3H, s), 5.88 (1H, s), 7.06 (1H,s), 7.95 (1H, bs),.10.21 (1H, s), 12.52 (1H, bs), 18.77 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.2, 10.9, 18.5, 19.4, 27.1, 27.8, 32.1, 32.4, 33.3, 44.0, 48.0, 52.6, 59.4, 97.2, 97.5, 103.0, 104.2, 105.1, 108.9, 143.4, 151.1, 151.5, 156.7, 165.5, 167.6, 180.8, 191.6, 198.1, 201.3; HRMS *m*/*z* 549.1917 (calcd for $C_{29}H_{31}O_6N_3S$, 549.1928).

Real-Time Detection of Tdp1 Activity. A fluorophore quencher-coupled DNA biosensor for real-time measurement of Tdp1 cleavage activity was recently designed in our laboratory.[42] The biosensor is a 16-mer single-stranded oligonucleotide containing both a 5′-FAM fluorophore donor and a quenching 3′-BHQ1 moiety.

Recombinant protein Tdp1 was expressed in *Escherichia coli* (pET 16B plasmid containing Tdp1 cDNA was provided by Dr. K. W. Caldecott, University of Sussex, UK) and isolated as described.[43,44] The reaction mixture in a final volume of 200 μL contained a Tdp1 reaction buffer (50 mM Tris-HCl, 50 mM NaCl, and 7 mM β-mercaptoethanol), a 50 nM oligonucleotide substrate, and varied concentrations of potential inhibitor. Purified Tdp1 was added in a final concentration of 1.5 nM.

The reaction mixtures were incubated at a constant temperature of 26 °C in a POLARstar OPTIMA fluorimeter (BMG LABTECH, GmbH, Germany), to measure fluorescence intensity each minute (Ex485/Em520 nm). Tdp1 inhibition was calculated by comparing the rate of increase in the fluorescence of a biosensor in the presence of a compound similar to that of DMSO (1.5%) control wells. The measurements were conducted in at least two independent experiments. IC_{50} values were determined using an 11-point concentration response curve. The data were imported into the MARS Data Analysis 2.0 software package (BMG LABTECH), and the slope during the linear phase (using data from 0 to 7 min) was calculated.

Cytotoxicity Assay. Analysis of the intrinsic toxicity of the compounds was conducted against the human cell line HeLa (cervical cancer), using the EZ4U cell proliferation and cytotoxicity assay (Biomedica, Austria), as per the manufacturer's protocols. Briefly, cells were grown in a Dulbecco's modified Eagle medium, using an antibiotic−antimycotic mixture (Gibco, Thermo Fisher Scientific, USA), and in the presence of 10% fetal bovine serum (Biolot) in a 5% CO_2 atmosphere. After formation of a 20−30% monolayer, tested compounds were added to the medium (the volume of added reagents was 1/100 of the total volume of the culture medium, and the amount of DMSO was 1% of the final volume), and the cell culture was monitored for 3 days. Control cells were grown in the presence of 1% DMSO. Further, an EZ4U (substrate) was added into each well, and cells were incubated at 37 °C for 2 h; absorbance was read at 450 or 492 nm, with 620 nm used as a reference, using a POLARstar OPTIMA fluorimeter. The compound concentration that caused 50% cell growth inhibition (CC_{50}) was determined using the OriginPro 8.6.0 software package. The measurements were conducted in three parallel experiments.

To study the influence of inhibitors on the cytotoxic effect of topotecan ("ACTAVIS GROUP PTC ehf."), the CC_{50} for Tpc was determined in the presence and absence of Tdp1 inhibitors. Enhancement of cytotoxity of Tpc was calculated as the ratio of CC_{50} for Tpc in the absence of Tdp1 inhibitor to CC_{50} for Tpc in the presence of 5 μM Tdp1 inhibitor.

Molecular Modeling. Models of the 3D structure of compounds **9b** and **9c** were obtained using the crystalline structure of UA[45,46] (CCDC ID: USNICA01) and using the molecular editor of the Molecular Operating Environment (MOE)[47] software, version 2019.01. Compound structures were optimized using an Amber10:-EHT (extended Hueckel theory) force field. The Tdp1 catalytic domain structure obtained at a resolution of 1.5 Å (PDB ID: 6N19)[48,49] was used for molecular docking. Docking was conducted using the Dock module of the MOE 2019.01 software. The structures of 30 complexes were calculated with the score parameter London dG; then, for five complexes, the structures of the complexes were refined with the score parameter GBVI/WSA dG. The analysis of the complexes was performed using the Ligand interactions module of the MOE 2019.01 software. The results were obtained using Institute of Automation and Control Processes of Far East Branch of Russian Academy of Sciences Shared Resource Center's "Far Eastern Computing Resource" equipment (https://cc.dvo.ru).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.9b01089.

^{1}H and ^{13}C NMR spectra of compounds **7e**,**g**; ^{1}H and ^{13}C NMR and mass spectra of compounds **9a**−**h** (PDF)

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Notes

The authors declare no competing financial interest.

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