Saniculoside N from *Sanicula europaea* L.

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Extracts from the aerial parts of *Sanicula europaea* L. were investigated for their anti-HIV activity, and the 50% ethanolic extract was shown to exhibit the highest activity. A new triterpene saponin glycoside, 21β-(angeloyloxy)-3-*O*-[β-D-arabinopyranosyl(1→4)-β-D-glucopyranosyl(1→3)-β-D-glucuronopyranosyl propyl ester]-3β,15,16,22α,28β-pentahydroxy-Δ^{12}-oleanene, saniculoside N (**1**), in addition to the known phenolic acids, rosmarinic acid (**2**), and caffeic acid (**3**) were isolated as major components. Rosmarinic acid was established as the principal active substance.

Sanicula europaea L. (Apiaceae) has been used as a traditional medicinal plant in the treatment of wounds, dermatological, gastrointestinal, upper airway, and urinary system diseases, and chronic chest pains.[1] It was recently reported that aqueous extracts of *S. europaea* exhibit significant antiphage[2,3] and *in vitro* virucidal[4] effects in the phage−bacteria system, and antiinfluenza[5] activity against influenza A. It was established that saponins are responsible for antimicrobial,[6] hemolytic,[7] and antioxidant[8] activities, whereas hydroxycinnamic derivatives, especially rosmarinic acid, were responsible for antioxidant activity.[9,10] It was also reported that the presence of allantoin raised the value of the folk medicinal use of the plant.[11]

Bioassay-directed fractionation of the aerial parts of *S. europaea* L., grown in Northeast Turkey, led to the isolation of rosmarinic acid (**2**) as the most active constituent against HIV-1. In addition, a new saponin, saniculoside N (**1**), and caffeic acid (**3**) were isolated as major components, and the aglycon of the new saponin, saniculagenin N (**1a**) was obtained as a product of acidic hydrolysis of **1** (Chart 1).

The 50% EtOH extract of *S. europaea* (see the Experimental Section) showed the highest activity (99.5% inhibition at 200 μg/mL) and the lowest inhibitory concentration (IC_{50} = 39.0 μg/mL) in the HIV-RT assay, and this extract was thus fractionated by column chromatography and the fractions tested against HIV-1 (Table 1). The most active fractions, 3−5, were combined and the mixture was separated by MPLC to yield the new triterpene saponin glycoside, saniculoside N (**1**). The remaining major components were two hydroxycinnamic derivatives, rosmarinic acid (**2**) and caffeic acid (**3**), of which **2** was the major active compound.

The structure of **1** was established by analysis of spectral data and by chemical reactions. The IR (ν_{KBr}) spectrum showed absorptions at 3400 (OH), 1730 and 1570 (ester), 1410, 1250 and 1160 (CO) cm^{-1}. The positive ion FAB-MS exhibited a peak at *m/z* 1147 [M + 2Na]$^+$, compatible with the molecular formula $C_{55}H_{88}O_{22}$. The negative FAB-MS showed a molecular ion peak at *m/z* 1099 [M − H]$^-$, in addition to peaks at *m/z* 967 [1099 − arabinose]$^-$, 805 [967 − glucose]$^-$, and 587 [805 − glucuronic acid propyl ester]$^-$, which indicated the sequence of the sugar chain. The ^{1}H NMR spectrum of **1** displayed the anomeric proton signals of the monosaccharides at δ 5.48 (1H, d, J = 9 Hz, for glucose), 5.81 (1H, d, J = 10 Hz, for arabinose), and 4.94 (1H, d, J = 7.6 Hz, for glucuronic acid) which led to the assignment of the anomeric configuration of the monosaccharides as β. In addition, multiplets between δ 3.40 and 4.10 indicated the presence of the CH_2O and CHO groups of the monosaccharides. The broadened q at δ 6.06 and the methyl signals at δ 1.92 (3H, d, J = 7 Hz) and 1.87 (3H, br s) showed the presence of an angelic acid ester in the molecule. The signals appearing at δ 3.23 (H-3, m) and 5.46 (H-12, br s) and the methyl signals between δ 0.87−1.07 and δ 1.30−2.10 suggested a R_1-barrigenol derivative.[12,13] The downfield shift of the glycoside C-3 carbon (δ 91.6) in comparison with C-3 of the aglycon (δ 77.97) indicated the saccharide chain linkage at C-3.[12] The interglycosidic linkages in the saccharide chain were suggested from their ^{13}C NMR spectra (Table 2).

The aglycon saniculagenin N (**1a**), obtained after acidic hydrolysis of the saponin glycoside, afforded an improved ^{1}H NMR spectrum. The signals belonging to the angelic acid ester appeared at δ 6.12 (H-3′, qq, J = 7, 1.5 Hz), 1.83 (H-4′, br d, J = 7 Hz), and 1.72 (H-5′, br s). The signals at δ 4.31 (br s, $J_{1/\omega}$ = 4 Hz), 4.04 (br s, $J_{1/\omega}$ = 4 Hz), 3.92 (d, J = 10 Hz), 3.54 (d, J = 12 Hz), and 3.21 (d, J = 6 Hz) indicated the presence of five hydroxyl groups in the molecule, and the signal at δ 5.40 (d, J = 10 Hz) was assigned to the methine ester proton. Irradiation of the ester proton at δ 5.40 (H-21) collapsed the doublet at δ 3.92 (H-22) to a singlet and vice versa, and irradiation of the signal at δ 3.54 (H-28) collapsed the doublet at δ 3.31 (H-28′) to a singlet. Irradiation of the signal at δ 6.12 (H-3′) collapsed the br doublet at δ 1.83 (H-4′) to a br singlet and sharpened the br s at δ 1.72 (H-5′). Two mutually coupled methine protons could be ascribed to H-15β and H-16β based on the small coupling constants, and this assignment was supported by the NOE enhancements observed on irradiation of

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Chart 1

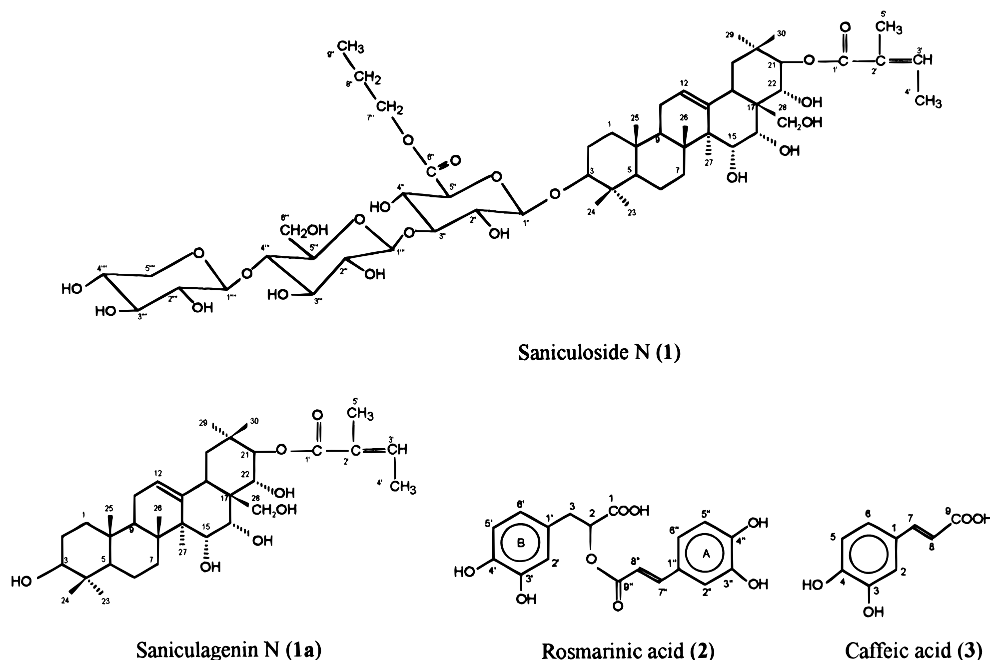

Table 1. HIV-1 RT Bioassay of the 50% EtOH Extract of *Sanicula europaea* L., and of the Fractions and Pure Substances Obtained from This Extract

sample[a]	inhibn%[b]	activity
50% EtOH extract	99.5	very active
1 (H_2O)	21	inactive
2 (10% MeOH)	59.8	moderately active
3 (30% MeOH)	79.1	active
4 (50% MeOH)	81.5	active
5 (70% MeOH)	76.3	active
6 (100% MeOH)	33.8	inactive
saniculoside N (**1**)	20.1	inactive
rosmarinic acid	57.4	moderately active
caffeic acid	0	inactive

[a] Fraction numbers as indicated with elution solvent in parentheses. [b] Inhibition % at 200 μg/mL

Table 2. ^{13}C NMR (APT) Chemical Shifts (ppm) of 1 in CD_3OD at 50.32 MHz

aglycon moiety		monosaccharide moieties						
			C-1	C-2	C-3	C-4	C-5	C-6
C-3	91.6							
C-12	127.0	Gluc a[a]	105.4	75.0	86.6[b]	72.7	76.2	156.0
C-13	143.6	Glu[a]	104.8	74.7	78.0	78.9[b]	78.3	63.6
C-15	77.1	Ara[a]	103.3	70.0	73.0	68.5	64.4	–
C-16	80.0							
C-21	56.8							
C-22	82.1							
C-28	67.8							
C-1′	174.1							
C-3′	138.0							

[a] Gluc a, glucuronic acid; Glu, glucose; Ara, arabinose. [b] Connection point to the next sugar moiety.

H_2-28 (12.5% to H-15β and 6.5% to H-16β).[14] Similarly, a large coupling constant allowed another, mutually coupled pair of methine protons (*trans* relationship) at δ 5.40 (d, J = 10 Hz), and δ 3.92 (d, J = 10 Hz) be ascribed to H-21α and H-22β or vice versa. The latter proton signal was assigned H-22β since a significant NOE enhancement (7.5%) was observed upon irradiation of H_2-28. Thus, the former signal was assigned to H-21α, which was shifted downfield by the angeloyl group. This proton in turn showed a NOE enhancement on the methyl group resonating at δ 1.36. These

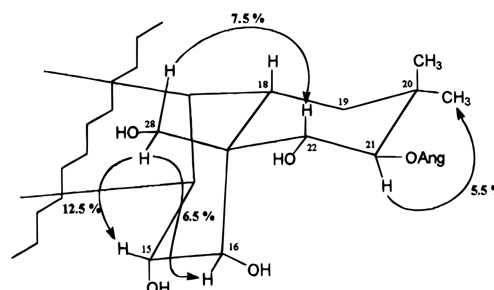

Figure 1. Partial structure of the aglycone from DQF-COSY and NOE difference experiments.

interactions are shown in the partial structure of the aglycon (Figure 1). Thus, the structure of the aglycon was determined as 21-angeloyl-R_1-barrigenol, for which only ^1H NMR data in $CDCl_3$ were originally reported.[15] More recently, its ^{13}C NMR data (in $CDCl_3$) were published with some ambiguity and uncertainty.[16] The ^{13}C NMR data of **1a** (see the Experimental Section) were assigned by analogy to related compounds and with the aid of APT, DQF-COSY, and HMQC spectra in this study.

The CI mass spectrum of the aglycon gave a molecular ion peak at m/z 589.3 $[M + H]^+$, and the predominant fragmentation ion m/z 471 $[M + H - 18 - 100]^+$ due to the expectedly easy loss of one molecule of water and angelic acid, corresponding the molecular formula $C_{35}H_{56}O_7$ (MW 588). High-resolution mass spectrometry yielded a molecular peak at m/z 588.4023. The EI-MS of the compound showed the retro-Diels–Alder fragments (Figure 2) at m/z 207 ($C_{14}H_{24}O$) and 380 ($C_{21}H_{32}O_6$), and fragment ions at m/z 362 $[380 - H_2O]^+$ and 280 $[380 - \text{angelic acid}]^+$ supporting the $\Delta^{12,13}$-unsaturation in the molecule. All of the spectral data of the new saponin and its aglycon revealed differences from those of the saponins reported formerly.[17-19]

The structures of the known compounds rosmarinic acid and caffeic acid were identified via spectral data and TLC comparisons with authentic samples.[20,21]

Antiviral activity tests on the pure substances showed that the main active component was rosmarinic acid;

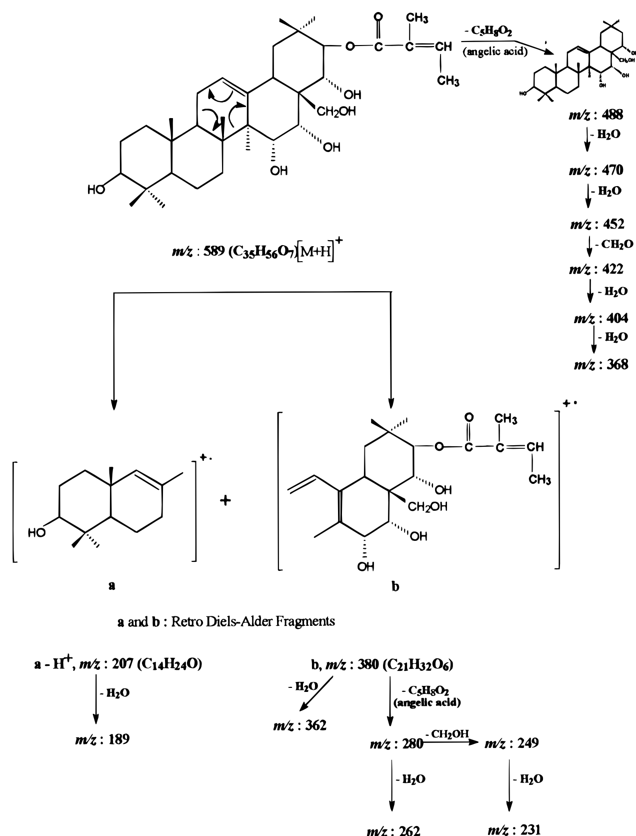

Figure 2. The EI-MS fragment ions of saniculagenin N (1a).

this compound showed weak antiviral activity, with 57.4% inhibition in the HIV-RT system at 200 μg/mL. Caffeic acid and saniculoside N exhibited no significant antiviral activity.

Experimental Section

General Experimental Procedures. IR spectrum (ν_{max} cm^{-1}) was taken on a Perkin-Elmer 983 spectrophotometer in KBr tablets. ^{1}H NMR spectra were recorded on a Bruker AC-200 L instrument operating at 200 MHz, and ^{1}H, COSY, NOE, DQF-COSY, and HMQC experiments were performed on a Varian XL 300 instrument and/or GE OMEGA 500 spectrometer. ^{13}C NMR (APT) spectra were run either at 50.32 MHz in CD_3OD and $CDCl_3$ or at 300 MHz in C_5D_5N and TMS was used as internal standard. FAB-MS, CI-MS, HR-MS, and EI-MS were recorded on a VG Zabspec spectrometer using [thioglycerol + Na$^+$] as a matrix. Optical rotations were measured on a Schmidt Haensch Polartronic E polarimeter in MeOH. Merck DC Alufolien Kieselgel 60F$_{254}$ plates were used for TLC; Kieselgel 60 G (100 g) mixed with Kieselgel 60 HF 254 (50 g) suspension in distilled water (350 mL) was used to prepare the preparative layers (20 × 20 cm plates in 0.5 mm thickness); and polyclar AT (polyvinylpolypyrrolidone) manufactured by GAF Corp. and Merck Lichroprep RP-18 for the column chromatographic separations.

Plant Material. *S. europaea* L. was collected from Arhavi (Northeast Turkey) in August 1993. A voucher specimen (No. 864) was deposited in the Herbarium of the Section of Botany, Department of Biology, Faculty of Science, Istanbul University. The plant material was identified by Prof. Dr. A. Aydın.

Extraction. The air-dried aerial parts of the plant material (940 g) were powdered and extracted with 50% EtOH (6 L) in a water bath (65 °C) for 1 h. In addition, a sample of the plant material (5 g) was extracted with 70% EtOH in a Soxhlet for 8 h. The aerial parts of the plant material (750 g) were also extracted with $CHCl_3$ (5 L) and 96% EtOH (5 L) successively at room temperature. Each extract was concentrated by vacuum evaporation. In this way, four different crude extracts were obtained: a 50% EtOH extract, a 70% EtOH extract, a $CHCl_3$ extract, and a 96% EtOH extract.

Isolation Procedures. Percentage inhibition values at 200 μg/mL of the $CHCl_3$, 96%, and 70% EtOH extracts were 47.1, 6.0, and 72.6, respectively. As the 50% ethanolic extract was found to show the highest antiviral activity against HIV-1 reverse transcriptase, isolation procedures were carried out only on this extract. The extract was separated chromatographically by two different methods. Method 1 yielded the pure saponin glycoside (**1**) whereas method 2 afforded the phenolic acids, rosmarinic acid (**2**) and caffeic acid (**3**).

Method 1. The 50% ethanolic extract (19.1 g) was suspended in H_2O and subjected to an open column prepared with polyvinylpolypyrrolidone (polyclar) in 50% MeOH and eluted with H_2O:MeOH (100:0 → 0:100). The fractions that showed activity in the HIV-RT assay (Table 1) were further purified by MPLC according to the method reported formerly,[22] using Lichroprep RP-18 (Merck) adsorbent, H_2O:MeOH gradient (50:50 → 0:100), and DMSO as the injection solvent. A pure saponin glycoside, saniculoside N (**1**, 200 mg), was obtained in this separation. All fractions were analyzed by TLC on silica gel plates in $CHCl_3$-MeOH−H_2O (61:32:7). Detection was performed by spraying with 30% H_2SO_4 in H_2O, followed by heating at 120 °C, for 5−10 min.

Method 2. The precipitation technique[9] was used in order to remove the saponins from the extract and obtain the hydroxycinnamic derivatives. The crude extract (25 g) was treated with a 5-fold volume of cold ether, and the precipitate was removed from the liquid phase and suspended in MeOH. Five volumes of Me_2O were added to the suspension. The precipitate was filtered off, and the filtrate was evaporated. The dried liquid phase was dissolved in distilled water and extracted with Et_2O and EtOAc, successively. The organic phases were separated and purified by preparative layer chromatography using Kieselgel 60 G mixed with Kieselgel 60 HF 254 as adsorbent and $CHCl_3$−MeOH−H_2O (61:32:7) as eluent to yield pure rosmarinic acid (24 mg) and caffeic acid (11.7 mg). These substances were visualized with 3% $FeCl_3$ reagent on TLC and PLC plates.

Saniculoside N (1): $[\alpha]^{18}_{D}$ −89.9° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3400 (OH), 2970, 2920, 2880, 2850 (CH), 1730, 1570 (COO), 1410, 1250, 1160 (CO) cm^{-1} ; ^{1}H NMR (CD_3OD, 200 MHz) δ 0.87−1.07 (each 3H, s, 7 × aglycon CH_3), 1.30−2.10 (ester CH_3, CH and CH_2), 3.23 (1H, m, α proton of H-3), 3.40−4.10 (aglycon CH_2O and CHO), 4.94 (d, *J* = 7.6 Hz, anomeric proton of glucuronic acid), 5.46 (1H, br s, H-12), 5.48 (d, *J* = 9 Hz, anomeric proton of glucose), 5.81 (d, *J* = 10 Hz, anomeric proton of arabinose), 6.06 (1H, br q, H-3′ of angelic acid) ; ^{13}C NMR (CD_3OD, 50.32 MHz) see Table 2; positive ion

FAB-MS m/z [M + 2Na]$^+$ 1147 (calcd for $C_{55}H_{88}O_{22}$, 1100); negative ion FAB-MS m/z [M]$^-$ 1099.

Acid Hydrolysis of 1. Acid hydrolysis was carried out as previously reported.[23] Saponin (15 mg) was dissolved in MeOH-benzene (5 mL, 1:1), and 2 N HCl (5 mL) was added to the solution. The mixture was refluxed for 6 h. The organic layer was evaporated *in vacuo*, and distilled water was added to the residue. The aglycon was extracted with $CHCl_3$. Monosaccharide analysis was performed using the technique of acidic hydrolysis on a TLC plate.[24] The saponin glycoside and the sugar references were applied on a TLC layer (silicagel HF 254) and treated with concentrated HCl vapor in a closed vessel saturated with the acid vapor for 15 min at 100 °C. After the vessel was cooled and the acid vapor was removed from the plate, TLC was performed using the lower phase of $CHCl_3$−MeOH−H_2O (65:30:10 + 10% MeOH) as eluent, and the compounds were detected with anisaldehyde.

Saniculagenin N (1a): $[\alpha]^{18}_D$ +27.0° (c 0.1, MeOH); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.79−1.34 (s, each 3H, H-23−H-27), 1.00 (s, each 3H, H-29 and H-30), 1.72 (bs, H-5′), 1.83 (bd, J = 7 Hz, H-4′), 3.22 (dd, J = 6.0, 6.0 Hz, H-3), 3.31 (d, J = 12 Hz, H-28′), 3.54 (d, J = 12 Hz, H-28), 3.92 (d, J = 10 Hz, H-22), 4.04 (bd, J = 4 Hz, H-16), 4.31 (bs, H-15), 5.37 (bs, H-12), 5.40 (d, J = 10 Hz, H-21), 6.12 (q, J = 7.0, 1.5 Hz, H-3′); ^{13}C NMR (C_5D_5N, 300 MHz), δ 168.50 (C-1′), 144.37 (C-13), 136.09 (C-3′), 129.52 (C-2′), 124.84 (C-12), 81.35 (C-21), 77.97 (C-3), 72.57 (C-16), 72.37 (C-22), 67.46 (C-15), 65.25 (C-28), 55.55 (C-5), 48.42 (C-19), 47.94 (C-17), 47.39 (C-14), 47.13 (C-9), 41.42 (C-8), 41.27 (C-18), 39.25 (C-4), 37.36 (C-20), 36.75 (C-10), 36.07 (C-7), 29.86 (C-27), 29.79 (C-23), 29.71 (C-29), 29.31 (C-1), 28.15 (C-2), 23.99 (C-11), 21.08 (C-4′), 20.32 (C-30), 19.08 (C-6), 17.57 (C-26), 16.61 (C-25), 15.91 (C-24), 15.87 (C-5′); ^{13}C NMR (APT in $CDCl_3$ at 50.32 MHz), δ 142.4 (C-13), 138.0 (C-3′), 125.6 (C-12), 80.7 (C-21), 71.2 (C-16), 67.0 (C-28); CI-MS m/z [M]$^+$ 589 (15), [M − angelic acid]$^+$ 489 (13), [M − angelic acid − H_2O]$^+$ 471 (100) ; EI-MS m/z [M]$^+$ 588 (2), [M - H_2O]$^+$ 570 (11), [M − angelic acid]$^+$ 488 (11), [retro-Diels−Alder fragment **a** $C_{14}H_{24}O$ − H]$^+$ 207 (94), [retro-Diels−Alder fragment **b** $C_{21}H_{32}O_6$]$^+$ 380 (9) and the other fragments at m/z 470 (22), 452 (17), 422 (51), 404 (21), 368 (10), 362 (14), 280 (66), 262 (25), 249 (53), 231 (68), 189 (98), see also Figure 2; HRMS m/z [M]$^+$ 588.4023 (100).

HIV-RT Bioassay. The activity tests were performed as previously reported.[25] The assay mixture contained 50 mM Tris·HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethyleneglycolbis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 5 mM dithiothreitol, 0.3 mM glutathione, 2.5 μg/mL bovine serum albumin, 41 μM poly(rA) {$\in$260 (mM) = 7.8}, 9.5 μM oligo (dT) {$\in$260 (mM) = 5.6}, 20 μM TTP and 0.5 μCi [^{3}H]TTP. The reaction was started by the addition of 10 μL of HIV-1 RT (purified HIV-1 (p66/p51) reverse transcriptase), and the mixture was permitted to incubate at 37 °C for 1 h. Reaction was terminated by the addition of 25 μL of 0.1 M EGTA followed by chilling on ice. Aliquot of the reaction mixture was then spotted uniformly onto circular 2.5 cm DE-81 (Whatman) filters, kept an ambient temperature for 15 min and washed four times with 5% aqueous $Na_2HPO_4 \cdot 7H_2O$. This was followed by two more washings with doubly distilled H_2O. Finally, the filters were thoroughly dried and subjected to scintillation counting. For testing enzyme inhibition, five serial dilutions of samples in DMSO were added to the reaction mixtures prior to the addition of enzyme. The median inhibitory concentration (IC_{50}) was calculated from a linearly regressed dose−response plot of percent control activity vs concentration of compound, utilizing at least five concentrations of each compound.

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