Combining Fundamental Kinetics and Standard Alkylation Assays to Prioritize Lead-like KRAS G12C Inhibitors

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Supplemental Information

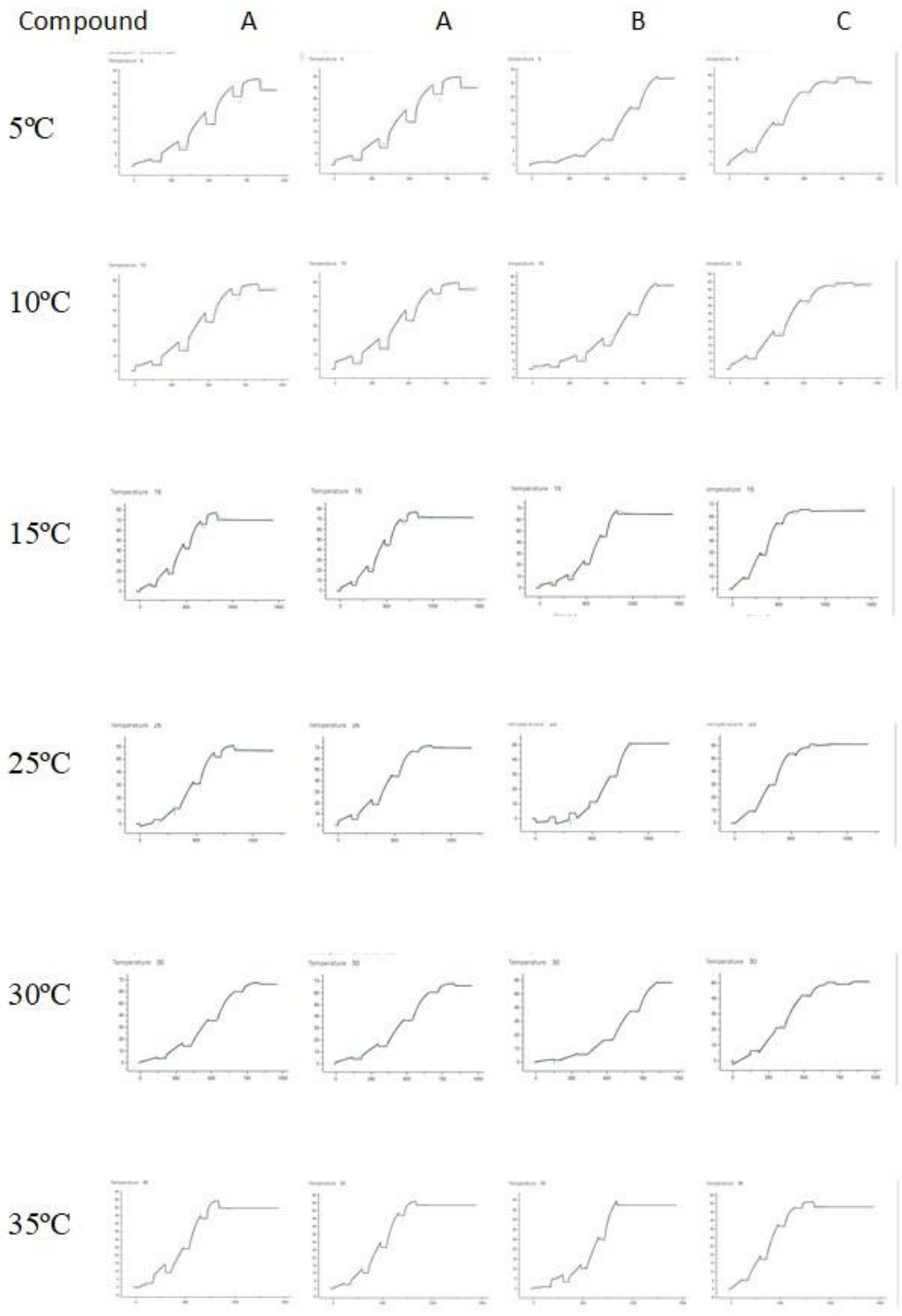


Figure S1: Measured SCK titration curves (green) for analysis of compounds A, B and C alkylating KRAS G12C at 5 °C, 10 °C, 15 °C, 25 °C, 30 °C and 35 °C. SCK curves were fit (black) to Reaction (4) assuming pseudo-first-order irreversible binding kinetics. Compound A was run in duplicate. The fit returned k_{alk} and the results are shown in Table S1. Repeating the assay run at multiple temperatures required a fresh sensor chip per temperature since alkylation was irreversible thereby preventing reuse.

Table S1: Parameter values returned from fitting Reaction (4) as shown in Figure S1. Standard error of the fit associated with k_{alk} is SE_k_{alk}, standard error of the fit associated with R_{max} is SE_R_{max}
The global relative χ^2 = Average (χ^2 /R_{max}) = 0.015 ± 0.01

Temperature (°C)	Compound	k_{alk} ($M^{-1}s^{-1}$) x 10^4	SE_k_{alk} ($M^{-1}s^{-1}$) x 10^4	R_{max} (RU)	SE_R_{max} (RU)	χ^2 (RU^2)	Relative χ^2 (RU)
5°C	A	4.27	0.0146	34.86	0.03	0.94	0.03
5°C	A	4.18	0.0132	31.82	0.02	0.67	0.02
5°C	B	11.2	0.0246	32.15	0.01	0.35	0.01
5°C	C	1.63	0.00164	33.55	0.01	0.05	0.001
10°C	A	4.94	0.0150	54.96	0.04	1.97	0.04
10°C	A	4.87	0.0131	53.73	0.03	1.49	0.03
10°C	B	8.57	0.0188	53.09	0.02	1.00	0.02
10°C	C	1.60	0.00456	52.75	0.04	0.94	0.02
15°C	A	5.13	0.00800	71.57	0.02	1.55	0.02
15°C	A	4.82	0.00777	70.10	0.02	1.58	0.02
15°C	B	9.61	0.00859	64.61	0.01	0.41	0.01
15°C	C	1.90	0.00354	67.23	0.02	1.36	0.02
25°C	A	5.19	0.00818	69.92	0.02	1.01	0.01
25°C	A	4.08	0.00665	56.91	0.01	0.69	0.01
25°C	B	11.1	0.0110	60.93	0.01	0.30	0.00
25°C	C	1.12	0.00469	58.94	0.08	1.32	0.02
30°C	A	4.22	0.00482	66.05	0.02	0.36	0.01
30°C	A	3.99	0.00472	66.32	0.02	0.39	0.01
30°C	B	9.12	0.0151	50.67	0.02	0.48	0.01
30°C	C	1.55	0.00187	62.17	0.02	0.18	0.00
35°C	A	3.55	0.00551	53.81	0.02	0.40	0.01
35°C	A	3.45	0.00816	49.79	0.02	0.80	0.02
35°C	B	7.57	0.0141	47.97	0.02	0.48	0.01
35°C	C	1.30	0.00323	46.95	0.04	0.36	0.01

Table S2: Kinetic parameters estimated from Figure 4A.

T (K)	k_{on} ($M^{-1}s^{-1}$) ± SE x 10^5	k_{off} (s^{-1}) ± SE	k_{inact} (s^{-1}) ± SE	Ln(k_{on}/T) ± SE	Ln(k_{off}/T) ± SE	Ln(k_{inact}/T) ± SE
278	2.15 ± 0.001	0.187 ± 0.01	0.0196 ± 0.0001	6.650 ± 0.0046	-7.30 ± 0.052	-9.56 ± 0.003
288	2.71 ± 0.001	0.557 ± 0.04	0.0419 ± 0.0002	6.846 ± 0.0037	-6.25 ± 0.069	-8.84 ± 0.005
298	2.19 ± 0.003	1.271 ± 0.01	0.151 ± 0.0001	6.599 ± 0.0136	-5.46 ± 0.008	-7.59 ± 0.001

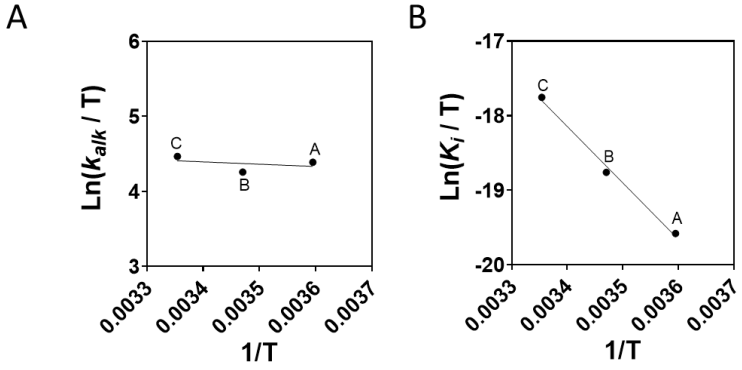


Figure S2. Temperature dependence of k_{alk} and K_i for compounds A, B and C fit to a simple line. For k_{alk} a near flat line indicates little temperature dependence while K_i increased with temperature as indicated by the linear fit where Ln(K_i/T) = 7.540-7554/T, R^2 = 0.99.

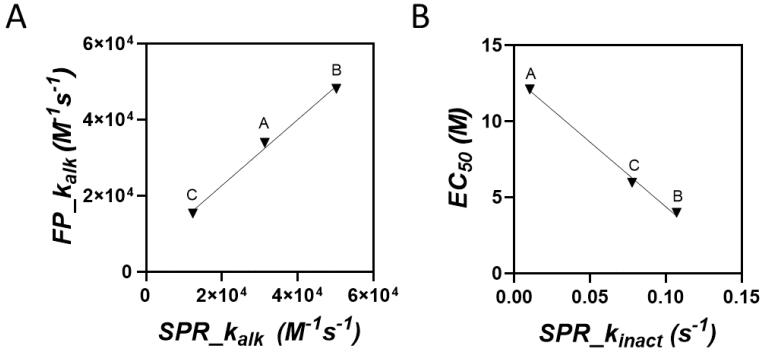


Figure S3. Linear correlation of SPR parameters for compounds A, B and C with respect to results from orthogonal assays. (A) Linear k_{alk} correlation between SPR and FP assay, assumed to go through the origin, and fit returned slope = 1.00 (± 0.05) and R^2 = 0.96. (see Method S5). (B) The linear correlation of k_{inact} versus cell EC_{50} returned slope = -85 (± 5) and R^2 = 0.99.

Equation S1: Eyring Transition State Analysis

The Eyring equation[1] can be expressed as follows,

$$\mathbf{Ln}\left(\frac{k}{T}\right) = \left(\frac{-\Delta H^{\ddagger}}{R}\right).\left(\frac{1}{T}\right) + \left(\frac{\Delta S^{\ddagger}}{R}\right) + \mathbf{Ln}\left(\frac{k_B}{h}\right) \qquad \text{Equation (S1)}$$

where k is one of the three kinetic constants for the reaction, R is the ideal gas constant, k_B is the Boltzman constant, h is planks constant, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation at a molar standard state at 25 °C. The free energy of activation was calculated as $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T.\Delta S^{\ddagger}$. Estimates of $\Delta H^{\ddagger}$ $\Delta S^{\ddagger}$ and T. $\Delta S^{\ddagger}$ allowed the thermodynamic energy transitions to be plotted in Fig. 4C.

Table S3. Thermodynamic transition energies estimated from fit of data in Supplemental Table S1 to the Equation (S1) (Eyring equation).

	$\Delta G^{\ddagger}$ (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$T.\Delta S^{\ddagger}$ (kJ/mol)
k_{on}	42.35 ± 0.41	-1.58 ± 8.85	-43.93 ± 9.17
k_{off}	72.34 ± 0.18	63.76 ± 4.00	-8.57 ± 4.15
k_{inact}	77.96 ± 0.54	67.76 ± 11.78	-10.20 ± 12.20

Table S4. Parameter values for reaction coordinate plot in Fig. 4 estimated from parameter values in Supplemental Table S3.

Reaction Coordinate	ΔG (kJ/mol)	ΔH (kJ/mol)	-T.ΔS (kJ/mol)
P + L	0	0	0
$PL^{\ddagger}$	42.4 ± 0.4	-1.6 ± 8.8	43.9 ± 9.1
PL	-30.0 ± 0.6	-65.3 ± 4.0	35.3 ± 4.1
$PL_x^{\ddagger}$	47.9 ± 0.5	2.4 ± 11.7	45.5 ± 12.2

Energy transitions along the reaction co-ordinate (chosen standard temperature) for binding of compound A to KRAS G12C were obtained from Supplemental Table S3 as follows. Unreacted species have zero transition state energy and overcome a free energy barrier ($\Delta G^{\ddagger}$) possessing enthalpic ($\Delta H^{\ddagger}$) and entropic contributions ($T.\Delta S^{\ddagger}$). The transition state energies $\Delta H^{\ddagger}$ and $T.\Delta S^{\ddagger}$ are calculated first along the reaction coordinate which then allow the reaction coordinate for $\Delta G^{\ddagger}$ to be calculated from $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T.\Delta S^{\ddagger}$. The transition state energies $\Delta H^{\ddagger}$ and $T.\Delta S^{\ddagger}$ for the association process are taken directly from Eyring analysis (Supplemental Table S3) while the energy of the affinity complex PL, an equilibrium state, is then obtained by subtraction of the energy of the dissociation process from that of the association process. The energy of the transition state $PL_x^{\ddagger}$ towards the inactivated complex PL_x is the sum of the energy from the inactivation process and the energy of the affinity complex. Importantly, entropic energies must be expressed as negative energy (-T.ΔS) because entropy is a measure of system disorder and a decrease in system energy. The free energy associated with the stability of the adduct PL_x was not determined but formation of the covalent bond will have very high negative enthalpy.

Method S1. k_{inact}/K_i Measurements by SCK-based SPR Analysis

A series S SA sensor chip (Cytiva), which is supplied pre-coated with streptavidin, was docked in a Biacore 8K system and equilibrated in running buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl and 0.005% Tween20). The sample compartment temperature and analysis temperature were held constant at 5°C. KRAS G12C-Avitag (MW~22kDa) was recombinantly expressed in house and affinity captured giving capture responses of approximately 4500-5000RU on the working sensing spot while the reference sensing spot remained uncoated. Serial tripling dilutions of compound were prepared in running buffer and injected over this pair of sensing spots for 120s from low to high concentration for 200s at 100uL/min and were allowed to dissociate for 10 min. This was also repeated for blank injections and solvent correction was performed according to manufacturer's recommendations. The 8K Biacore allows parallel injections over enabling all test compound to be completed at one temperature set point using a single SPR chip. This entire procedure was repeated at six temperatures settings, 5°C, 10°C, 15°C, 25°C, 30°C and 35°C. In this analysis regime the binding curves are dominated by the accumulation of adduct with low contributions from the affinity component allowing the affinity component to be neglected. See Supplemental Method 4 for curve fitting, data plotting and statistics.

Method S2. Estimating Fundamental Kinetic Parameters

A series S SA sensor chip (Cytiva), which is supplied pre-coated with streptavidin, was docked in a Biacore S200 system and equilibrated in running buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl and 0.005% Tween20). The sample compartment temperature and analysis temperature were held constant at 5°C. KRAS G12C-Avitag (MW~22kDa) was affinity captured giving capture responses of approximately 4500-5000RU on the working sensing spot while the reference sensing spot

remained uncoated. 100nM compound prepared in running buffer was injected over this pair of sensing spots for 4s at 100uL/min with > 200s dissociation. This injection was immediately repeated for the same sample but with an extended contact time of 8s and a final replicate was performed for a constant time of 16s. These injections were bracketed by multiple blank injections to allow optimal double referencing. The serially increasing contact time result in increased accumulation of irreversible adduct. Data was imported into Biacore S200 evaluation software and fit to Reaction (3). See Supplemental Method 4 for curve fitting, data plotting and statistics.

Method S3. Transition State Analysis

A series S Protein G sensor chip (Cytiva), which is supplied pre-coated with Protein G, was docked in a Biacore S200 system and equilibrated in running buffer (50 mM HEPES, pH 7.5, containing 150 mM NaCl and 0.005% Tween20).

Affinity Capture Stack: At a flow rate of 10µL/min, injected 20mM Glycine, pH1.5, over all channels for 30s followed by injection of a 1/200 dilution of anti-streptavidin (Bio-Techne, Prod# NB120-10022) for 200s, followed by streptavidin (50µg/ml in running buffer) for 100s. KRAS G12C-Avitag was injected over the working sensing spot resulting in affinity capture of approximately 1300-1400RU while the reference sensing spot remained uncoated. The surfaces were allowed stabilize for > 5 min before injection of compound over both coated and uncoated (reference) sensing spots.

Sampling: Seven serial doubling dilutions of compound were prepared from 78nM to 5µM and injected in series at 100uL/min at contact times that decreased with increasing concentrations as follows 14s, 13.2s, 11.9s, 10.8s, 10.2s, 9.0s and 7.7s, respectively. Each sample was bracketed by a blank sample giving a total of three sample injections per tested concentration. Each test concentration was performed on a freshly prepared KRAS G12C surface. This entire sequence was automated and repeated at analysis temperatures of 5°C, 15°C and 25°C where the sample rack temperature was matched in each case. Data was imported into Biacore S200 evaluation software for model fitting. See Supplemental Method 4 for curve fitting, data plotting and statistics.

Method S4. Curve Fitting, Data Plotting and Statistics

The simulations in Fig. 1A and B were performed using BiaEvaluation Version 4.1 and are described in Fig. 1. The experimental SCK curves shown in Fig. 2A and in Supplemental Fig. S1 were fit to Reaction (4) using Biacore™ Insight Software (Version 5.0.18) returning the target inactivation rate constant, k_{alk} (units: $M^{-1}s^{-1}$). The kinetic data in Fig. 3 and Fig. 4A were fit to Reaction (3) using Biacore™ S200 Evaluation software, where k_r = constant = 0 returning k_f as the inactivation rate constant, k_{inact} (units: s^{-1}). All the above fitting programs were obtained from Global Life Sciences Solutions USA LLC (100 Results Way, Marlborough, MA 01752, USA). The energies on the reaction coordinate in Fig 4C were plotted using Graphpad. Prism version 10 (GraphPad Software, Boston, Massachusetts USA) and this program was also used in both Fig. 2B and Fig. 4B to fit a polynomial and Equation (S1), respectively. Specific details on how each data set was fit or plotted is provided in the relevant figure in each case. Curve fitting programs enable fitting of binding interaction data to interaction models by nonlinear regression in order to estimate parameter values and the associated statistical methods provide metrics that inform the goodness of fit and statistical confidence in the parameter estimates. The goodness of fit between a model curve and an experimental curve is best described by χ^2 when the number of data points is high (as in SPR curves) and by a regression coefficient R^2 when the number of values is low (e.g. Fig. 2B). χ^2 is the square of the averaged residual response difference and approaches the baseline noise for the best fits. The standard error of the fit (SE) associated with a given parameter returned from a model fit reports confidence in the parameter estimate. This form of SE is not related to the standard error of the mean for replicate values of a set of measurements. Rather, this form of SE is a measure of the information content of the data. In other words it assesses the degree to which the parameter value is defined and are related to parameter confidence intervals. Generally, we assume that values that have high SE indicates low confidence and often indicates overfitting.

Method S5. Alkylation Electromobility Shift Assays for Estimation of Cell EC_{50}.

The HCC1171 cells line were taken from the Genentech internal cell bank repository. Cells were grown under standard tissue culture conditions in RPMI media with 10% fetal bovine serum, 2% L-Glutamine, and maintained at 37°C, 5% CO_2 in a humidified incubator. The cells were seeded in 96-well plates at 20,000 cells/well in 70 µl of RPMI1640 media (Invitrogen) + 10 % Fetal Bovine Serum (Excell). Compound was added to the cells then next day at varied concentrations with a final DMSO of 0.3 %. After treatment of the cells for 18 h, cells were lysed by addition of 30 µl RIPA buffer

(Sigma) with 0.25 % SDS (Sigma). Plates were shaken for 30 min at 500 rpm at 4ºC, mixed three times with a multichannel pipette and spun at 2000 rpm for 15 min. Lysate from each well (20 µl) was added to 10 µl of 4x loading buffer (Invitrogen) and 3 µl of 10x reducing agent (Invitrogen) and then boiled for 10 min at 95° C. 15 µl of reduced/boiled lysate was loaded on a 4-20 %, 15-well TRIS-Glycine gel and run for 165 min and 110V and then transferred to a nitrocellulose membrane using iBlot Gell Tranfer Stacks (Invitrogen). The membrane was blocked for 1 h with blocking buffer at room temp (Rockland), and then incubated at 4°C overnight with KRAS antibody (Proteintech 12063-1-AP) diluted 1:1000 in blocking buffer. The next day, after several washes in TBST (Sigma), the membrane was stained with fluorescent anti-rabbit antibody (LI-COR 926-32211) for 1 h at room temperature in a black box. HCC1171 cells are homozygous for G12C so for every concentration there were two bands, a lower band representing the unalkylated G12C and an upper band represented alkylated G12C. The two bands are resolved because KRAS migrates around 21K while alkylated KRAS has a slightly higher molecular weight that results in a visible electrophoretic shift. IC50s are obtained by fitting an IC50 equation to an alkylation dose response curve calculated as the percentage modification for each concentration of a given compound. This is determined as the ratio of the intensity of the shifted band compared to the summed intensity of both bands (i.e. % alkylation = Alkylated / (Alkylated + non-alkylated). Following washes in TBS-T and TBS (Sigma), the membrane was imaged using a LI-COR Odyssey at medium resolution. Quantitate fluorescence intensity (FI) was measured over a rectangle encompassing a discrete band and Microsoft was used for data fitting.

Method S6. Fluorescent Polarization alkylation assay for Estimation of FP_k_{alk}

The fluorescein-labeled KR-Pep2d peptide (Ac-RRRRCPLYISYDPVCRRRR-NH2, FL-peptide)[51] was synthesized at Genentech. Binding assays were carried out in a 15 µL volume in a Proxiplate (Perkin Elmer) and fluorescence polarization was measured using an Envision plate reader (Perkin Elmer). Inhibitor titrations were dispensed to the assay plate using a D300 digital dispenser (Tecan) in a 300 nL total volume of inhibitor/DMSO (for 2% DMSO in the final assay volume). KRAS G12C was diluted to 50 nM in assay buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl2, and 0.01% Tween-20) containing 2 nM of FL-peptide and allowed to equilibrate for 30 minutes at room temperature. Following the equilibration period, the mixture of KRAS G12C and FL-peptide was added to the assay plate containing inhibitor/DMSO. Fluorescence polarization was monitored over an appropriate time course to capture the decrease in mP signal. Progress curve data was analyzed using DynaFit software (Biokin, Ltd.) to determine inhibitor k_{inact}/K_I with FL-Peptide binding kinetics held constant at experimentally determined values of k_{on} = 3.95 × 105 $M^{-1}s^{-1}$ and k_{off} = 0.0149 s^{-1}. For more details see also Figure S3.

Method S7. Cysteine Reactivity Assay

Incubation was carried out in 100 mM phosphate buffer (pH 7.4) at 37 °C with gentle shaking. Test compound (1 µM), diphenhydramine (0.1 µM, pre-internal standard), and cysteine (5 mM) were mixed sequentially to start the reaction. Aliquots were taken from 0, 30, 60, 90, 120, 150, and 180 min and quenched with 1x volume of ice-cold acetonitrile containing propranolol (2 µM, post-internal standard). The resulting samples were quantified via LC-MS/MS. Percentage remaining was calculated using peak area ratios normalized to the 0 min time-point sample. Half-life and k_{react} were then determined from fitting the data with one phase decay model ($\%_{remaining} = 100 * e^{-kreact * 5\,mM * time}$). Incubation without cysteine was also carried out to check compound stability in buffer.

Supplemental Results. Compound Purity

HLPS Analysis Configuration and Parameters

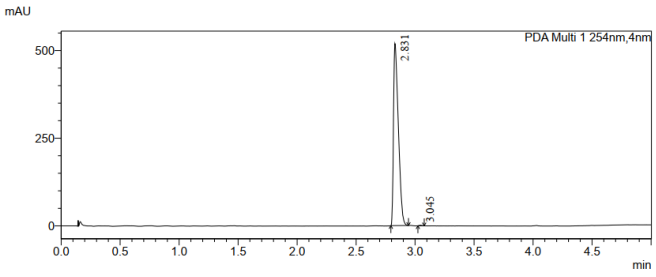
Comment	: Mobile phase A:Water/0.05%TFA; Mobile phase B:Acetonitrile/0.05%TFA

Instrument Name:Shimadzu LCMS-2020	
<<Pump>>	
Mode	: Binary gradient
Pump A	: LC-20AD
Pump B	: LC-20AD
Total Flow	: 1.2000 mL/min
B Conc.	: 5.0 %
<<Oven>>	
Oven Temperature	: 40 C
<<PDA>>	
PDA Model	: SPD-M20A
Lamp	: D2
Start Wavelength	: 190 nm
End Wavelength	: 400 nm

<<Interface>>	
Interface	:ESI
DL Temperature	:250 C
Nebulizing Gas Frow	:1.50 L/min
Heat Block	:250 C
Drying Gas	:On
	15.00 L/min
<<MS Parameter>>	
Initial Valve Position	:-
--Segment 1 Event 1--	
Start Time	:0.00 min
End Time	:5.00 min
Acquisition Mode	:Scan
Polarity	:Positive
Event Time	:0.50 sec
Detector Voltage	:+0.90 kV
Threshold	:0
Start m/z	:90.00
End m/z	:900.00
Scan Speed	:1667 u/sec
Interface Volt.	:Use the Data in the Tuning File
DL Volt.	:Use the Data in the Tuning File
Qarray DC Voltage	:Use the Data in the Tuning File
Qarray DC Voltage	:Use the Data in the Tuning File

Table S5. HPLC Results: The three compounds (A, B and C) were > 95% pure as shown in the HPLC chromatograms n in Figure S4-S6.

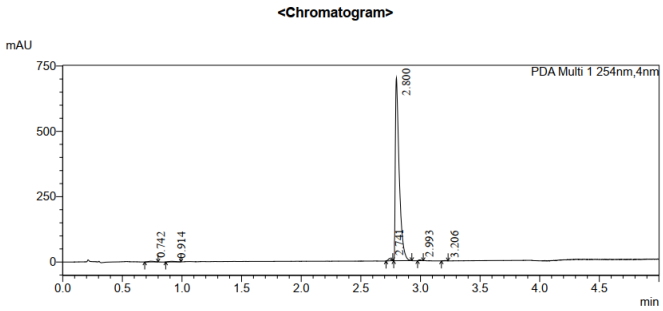
<Chromatogram>



Peak Table
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	2.831	517267	99.717	1483301	99.857
2	3.045	1469	0.283	2129	0.143
Total		518736	100.000	1485430	100.000

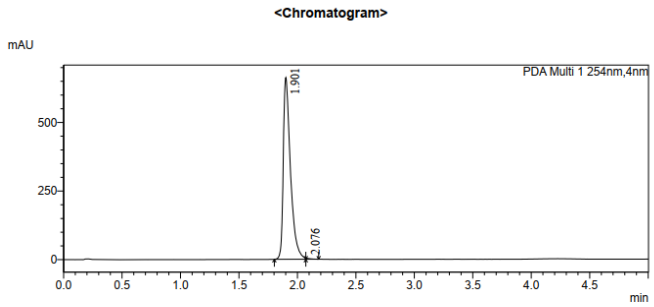
Figure S4. HPLC chromatogram for Compound A.



Peak Table
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	0.742	2901	0.418	8778	0.543
2	0.914	2284	0.329	9107	0.563
3	2.741	8794	1.268	13558	0.839
4	2.800	675810	97.413	1578955	97.672
5	2.993	2761	0.398	4204	0.260
6	3.206	1209	0.174	1982	0.123
Total		693760	100.000	1616585	100.000

Figure S5. HPLC chromatogram for Compound B.



Peak Table
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.901	662470	99.361	2826247	99.700
2	2.076	4258	0.639	8511	0.300
Total		666728	100.000	2834757	100.000

Figure S6. HPLC chromatogram for Compound C.

[1] Eyring, H. The Activated Complex in Chemical Reactions. Journal of Chemical Physics 1935, 3 (2), 107–115. https://doi.org/10.1063/1.1749604.