Electronic Supplementary Material (ESI) for Lab on a Chip

Integrated Microfluidics for Parallel Screening of Thousand *In Situ* Click Chemistry Reactions**

Supplementary information

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Fabrication of the microfluidic chips. The microfluidic chips were fabricated using a multilayer soft lithography method.[1, 2] Through photolithographic processes, two different silicon masters generated channels with either round (200 μm diameter, 40 μm high) or square (25 x 250 μm, 30 μm high) cross sections embedded in the respective layers of the poly(dimethyl-siloxane) (PDMS) matrix. Round channels (fluidic channels) were used for liquids; pressure or suction was applied to square channels (contol channels). The master utilized for fabrication of the fluidic channels was made by a one-step photolithographic process: a 35-mm thick negative photoresist (AZ 50XT) was spin-coated onto a silicon wafer (Silicon Quest, San Jose, CA). After UV exposure and development in AZ 400K developer (Clarian, Somerville, NJ), the surface profile of the patterned positive photoresist was transformed into a round profile by heating above the glass transition temperature of the positive photoresist (115^{o}C). The master for control channels was made by introducing a 30 μm-thin negative photoresist (SU8-2025, Microchem, Newton, MA) pattern on another silicon wafer. A valve is created when a fluidic channel crosses a contol channel. In order to achieve reliable performance of each valve, the widths of the control channels were set at 250 μm. Before fabricating, both the fluidic and control molds were exposed to trimethylchlorosilane (TMSCl) vapor for 2-3 minutes. A pre-mixed PDMS pre-polymer (GE, RTV 615 A

and B in 5 to 1 ratio) was poured onto the fluidic master located in a Petri dish to give a 6 mm-thick layer. Another portion of PDMS pre-polymer (GE, RTV 615 A and B in 20 to 1 ratio) was spin-coated onto the control master (1800 rpm, 60 s) to obtain the control layer. Both fluidic layer and control layer were cured in an 80 ^{o}C oven for 20 minutes. After incubation, the thick fluidic layer was peeled off the master, and holes (diameter 0.7 mm) were manually punched into the fluidic layer for access of reaction solutions. The fluidic layer was then trimmed, cleaned and aligned onto the thin control layer. After baking at 80 ^{o}C for 1 hour, the assembled layers were peeled off the control master, and another set of holes were punched for access of the control channels. The PDMS was bonded onto glass slides after oxygen plasma treatment (PDC-32G, Harrick Scientific, Pleasantville, NY) for 30s prior to contact. The chips were ready for use after baking at 80 ^{o}C for overnight.

Control Interface. The pneumatic control setup consists of 5 sets of eight-channel manifolds (Fluidigm, San Francisco, CA) controlled through a NI-DAQPad-6507 controller board (National Instruments Inc., Austin, TX) which was connected to a computer through a USB port. Nitrogen gas provided pressure (40 psi) to the manifolds. Forty control channels in the reaction circuit were first filled with DI-water and individually connected to the corresponding channels on the manifolds with metal pins (23 Gauge, New England Small Tube, Litchfield, NH) using Tygon microbore tubing (Cole-Parmer East, Bunker Court, IL). With activation of a regulator on the manifold, nitrogen gas entered the respective control line connected to the regulator, providing pressure to close valves in the microfluidic device. The control interface was created using a PC version of LabVIEW program (Version 8.0, National Instrument Inc. Austin, TX), which allows for manual control of individual valves and for automation of the synthetic processes.

General information. PDMS pre-polymers (RTV615 AB kit) were purchased from GE. Photoresist SU8-2025 was purchased from Clariant Corp. (Charlotte, NC). AZ50XT was purchased from AZ Electronic Materials (Somerville, NJ). All other chemical reagents were purchased from Acros, Lancaster and Sigma-Aldrich (St. Louis, MO) and were used as received. Solvents purchased from Aldrich were used without further purification. The acetylenes **I-VI** and azides **1-16** were kindly provided by Dr. H. C. Kolb (Siemens Medical Solutions Inc., Culver city, USA) besides the azides **6**. The azide **9** and the acetylene **VII** were provided by Prof. J. R. Heath from California Institute. The azide **6** was purchased from Aldrich. All acetylenes and azides were dissolved in DMSO/ethanol (1:4, v/v) to a final concentration of 6 mM. Bovine carbonic anhydrase (bCAII, Aldrich) was dissolved in PBS buffer (113 μM). The inhibitor **17**, ethoxazolamide[3] (K_d = 0.15 ± 0.03 nM) was dissolved in DMSO and then diluted 10-fold with a bCAII solution to give final concentrations of **17** and bCAII of 2 mM anc 113 μM. The Cu^I catalyst solution was composed with 0.15 mM $CuSO_4$, 3 mM sodium ascorbate and 0.06 mM tris((1-benzyl-1H-1,2,3-triazol-4- yl)methyl)amine in PBS (pH 7.4).

Details of general procedures. Through an computer-controlled interface, multiple steps (Fig. S1) were programmed using Labview software to generate each reaction mixture slug: (i) the acetylene I and azide 2 solutions were introduced simultaneously into the two parallel vertical channels in-between the two multiplexers, in 300 ms (flow rate: 67 nL/s); (ii) the acetylene and azide were pushed by back pressure into the rotary mixer within 200 ms with vacuum on (open filling[4]); (iii) depending on different reaction conditions, one of the bCAII, PBS buffer, Cu^I catalyst, or ethoxazolamide (inhibitor 17) in bCAII solutions was introduced into the rotary mixer within 5 s (dead filling[4]) to give a volume of 150 nL (flow rate: ~ 40 nL/s); (iv) the slug was circulated in the 150 nL rotary mixer using the mixing pump (pump

frequency: 100 ms) for 5 s; (v) additional PBS pushed the slug into the serpentine channel to give a total volume of 400 nL within 300 ms; (vi) the reaction mixture was driven into the PTFE tubing by back pressure within 1 s; (vii) all the fluidic channels were rinsed with PBS for 0.5 s, and then air for 0.5 s and this process was repeated once (total 2 s); (viii) one air (0.5 s) and one PBS slug (0.5 s, 400 nL) were introduced separately in *ca* 4 s to prevent cross contamination between two adjacent reaction mixture slugs. All steps were operated with the aid of a back pressure (nitrogen, 25 psi) and house vacuum to drive the operation/cycle. The flow rate for each step varies somewhat because it not only depends on the back pressure and the vacuum, but also depends on the volume of the various compartments (dead filling or open filling). All the reaction mixtures were sequentially generated and stored in 128 separate PTFE tubings which were placed in a moisture-regulated incubator at 37 °C for 40 h to complete the reactions. In theory, reaction mixture slugs could be stored indefinitely in PTFE tubing, but it proved to be convenient to store them in groups of 8/PTFE tube. The eight reaction slugs were expelled by the judicious application of positive pressure, with either water (10 uL) or guanidine HCl solution (10 μL, 0.1 M) into 200 μL microcentrifuge tubes.

SPE sample processing. The concept and procedures for the ZipTip® pre-purification process are described in Fig. S2. Each ZipTip® pipette tip contained C_{18} reversed-phase (C_{18}RP) resin (0.6 μL) and were used according to the manufacturer's recommendation except the adsorbed materials were eluted with water/acetonitrile/formic acid (50/50/.1, v/v/v, 20 μL, AWF).

ESI-MS/MS and ESI-MRM analysis. A Perkin-Elmer Sciex (Thornill, Canada) API III triple quadrupole mass spectrometer was tuned and calibrated in the positive ion mode of a mixture of polypropylene glycol (Mw 425, 1,000, and 2,000 (3.3×10^{-5}, 1×10^{-4}, and 2×10^{-3}M, respectively)) in

water-methanol (1:1, v/v) containing 2 mM ammonium formate and 0.1% MeCN. Normally, spectra were obtained by scanning at instrument conditions sufficient to resolve the isotopes of the polypropylene glycol-NH_4^+ singly charged ion at m/z 906 with 40% valley. To enhance sensitivity degraded resolution conditions were used in which the isotopes at m/z 906 were not resolved from one another. A stream of AWF was constantly infused into the ion source at 50 μL/min. Authentic standards (dissolved in AWF at 20 pmol/μL) or ZipTip® eluates were injected into this stream via a 20 μL injection loop. Mass spectra were recorded by scanning from m/z 100 to 700 (0.3 Da step size, about 6 s/scan, orifice 65 volts). Fragment ion spectra of Q1 (quadrupole number 1) preselected parent ions were recorded by scanning Q3 (quadrupole number 3) from m/z 50 to 700 (0.3 Da step size, about 6 s/scan, orifice 65 volts). Fragment ion spectra and MRM recordings were made with 10% nitrogen in argon collision gas with a collision gas thickness instrument setting of 130 and a rod offset (R0-R2) of 30 V.

To identify the hit compounds from the reaction mixtures, the detailed procedures are described in Fig. S3 using the reaction between **II** and **13** as an example: (i) ESI-MS in positive ion mode was used to identify the parent ion (P) from the Cu^I-catalyzed product which was pre-purified through the ZipTip® process (Fig. S3b); (ii) the tandem positive ion mass spectrum obtained from the same Cu^I-catalyzed product was used to record the fragment ion information (Fig. S3b). The most intense fragment ion (F) was selected as the signature for hit-identification; (iii) MRM (P→F transitions) was then used to screen the other reaction mixtures for the presence of a hit. The superior sensitivity of the MRM approach over other types of scans is a result of the dedicated recording of specific P→F transitions and the concomitant lowering of the background signal. Fig. S3c shows one experiment with 5 injections from the 5 different solutions (a) Cu^I, (b) bCAII with inhibitor, (c)-(d) bCAII in duplicate and (e) PBS. Besides the Cu^I-

catalyzed compound showing the signal, the bCAII-catalyzed products also show visible signals. Therefore, the reaction between **II** and **13** was identified as a hit-reaction. For all MRM recordings reported herein, degraded mass resolution conditions for both Q1 and Q3 were used to lower the limit for detection. Contemporary instrumentation with 10-100-fold increases in sensitivity over the Sciex API III^+ instruments would probably mean the same measurements could be made without the need to use degraded mass resolution conditions.

The remaining MRM results from the other samples are presented in Fig. S4. It is worthy to note that no reactions were observed between the acetylenes I, III-VIII and the azides 6 and 9, even in the presence of Cu^I catalyst.

References.

[1] M. A. Unger, H. P. Chou, T. Thorsen, A. Scherer, S. R. Quake, *Science* **2000**, *288*, 113-116.

[2] Y. N. Xia, G. M. Whitesides, *Annual Review of Materials Science* **1998**, *28*, 153-184.

[3] V. P. Mocharla, B. Colasson, L. V. Lee, S. Roper, K. B. Sharpless, C. H. Wong, H. C. Kolb, *Angewandte Chemie-International Edition* **2005**, *44*, 116-120.

[4] Dead filling means one valve at the end of the filling path is closed and the open filling means all the valves are open in the filling path. .

Captions to figures.

Fig. S1. Schematic diagrams summarize the generation of a single reaction slug which consisted of 15 steps in the circuit using the reaction between the acetylene **I** and the azide **1**. (A) The acetylene **I** and the azide **1** are introduced simultaneously into the two parallel vertical channels in-between the two multiplexers in *ca* 300 ms; (B) the acetylene and azide were pushed by air into the rotary mixer within *ca* 200 ms with vacuum i and ii on; (C) the bCAII solution was introduced into the rotary mixer within *ca* 5 s to give a volume of 150 nL with vacuum i on (dead filling); (D-E) slug circulates in the 150 nL rotary mixer using the mixing pump (pump frequency: 100 ms) for *ca* 600 ms; the right vertical channel in (A) is rinsed by PBS and air alternatively in *ca* 600 ms; (F) repeat (D) and (E) (total: 600 ms); (G) continue to circulate the solution in the rotary mixer for *ca* 4.8 s (total circulation time = (D + E + F + G), 6 s); (H) the bCAII is pushed with a PBS slug into the serpentine channel to give a total volume of 400 nL within *ca* 300 ms with vacuum ii on; (I) the reaction mixture is pushed into the PFTE tubing within *ca* 1 s with back pressure; (J-K) all fluidic channels rinsed thoroughly with PBS and air alternatively within *ca* 1 s; (L) repeat (J) and (K) (1 s); (M-N) one air and one PBS plug are introduced separately in *ca* 1.5 s to prevent cross contamination between two adjacent reaction slugs; (L) repeat M (500 ms).

Fig. S2. The cartoon shows the principle and procedures of the ZipTip® technique for prepurification of the triazole products.

Fig. S3. The identification process for hit compounds by ESI-MS, ESI-MS/MS, and ESI-MS/MS-MRM, using the compound II-13 as the example. (a) ESI-MS of the Cu^{I}-catalyzed reaction mixture showing the predicted parent ion at m/z 499.1 (calculated for $C_{23}H_{23}FN_6O_4S$: 499.1486). (b) The MS/MS spectrum derived from the m/z 499.1 parent ion in the Cu^{I} catalyzed reaction showing a major fragment ion at m/z

237.2. (c) MRM trace of the m/z 499.1→237.1 transition from 5 sequential injections (a-e) from: (a) Cu^{I}-catalyzed reaction; (b) bCAII with the presence of inhibitor catalyzed reaction ; (c-d) bCAII catalyzed reaction; (e) PBS control (no catalyst).

Fig. S4. (a)-(o). *In situ* click chemistry reactions between acetylenes **I-VIII** and azides **1-16**, analyzed by MRM**.** Some reactions between the acetylenes **I**, **III-VIII** and the azides **6**, **9** are missing because the triazoles cannot be generated from their Cu^{I}-catalyzed reactions. One MS spectrum include 5 injections (a-e) and each injection containing 8 samples if applicable which were prepared in the presence of Cu^{I} (a), bCAII and inhibitor (b), bCAII (c-d) and PBS only (e).

Table S1. Summary of the comparison between the conventional 96-well approach and the two generations of microfluidic platforms.

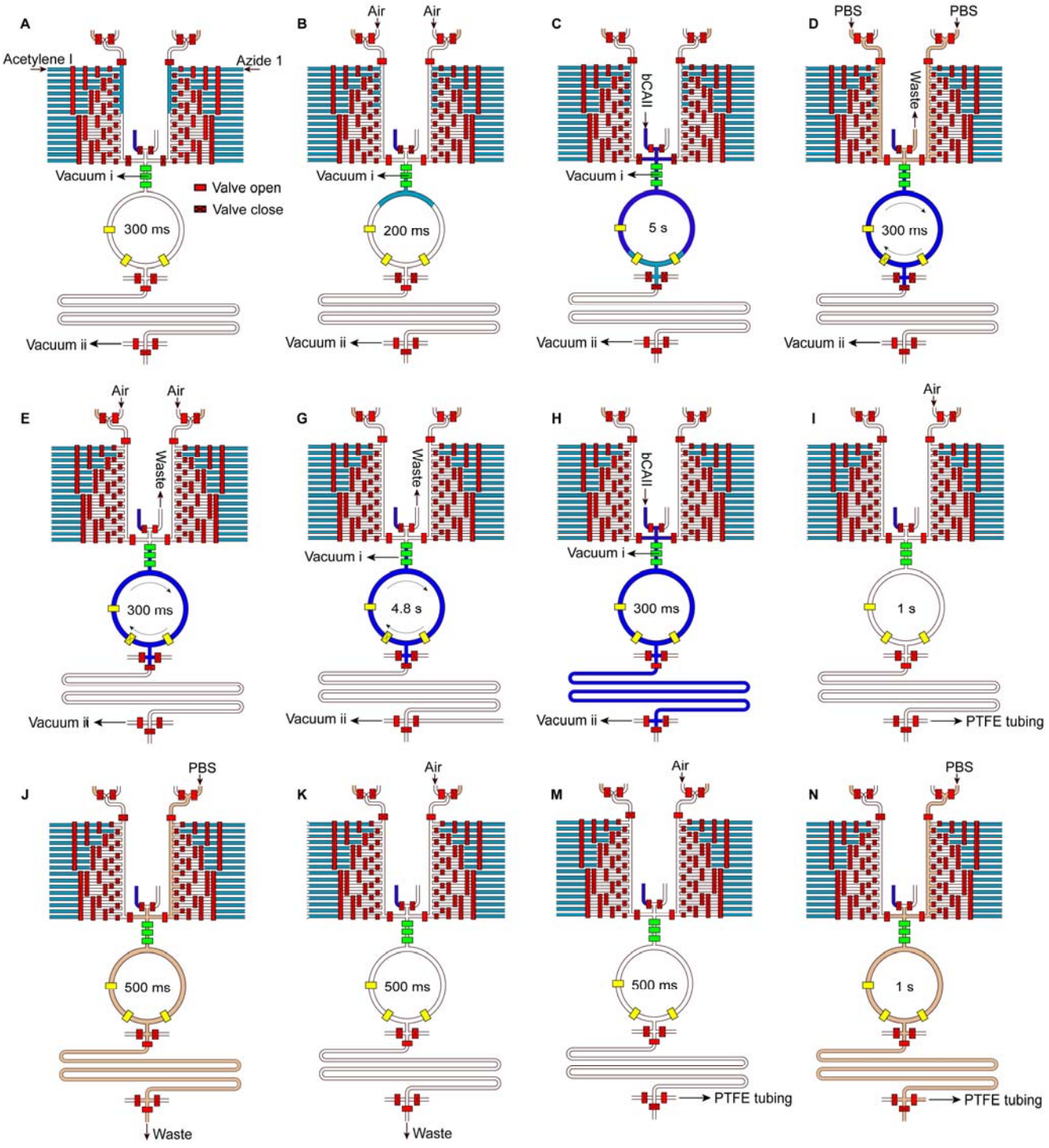

A
Acetylene I
Azide 1
Vacuum i
Valve open
Valve close
300 ms
Vacuum ii
B
Air
Air
Vacuum i
200 ms
Vacuum ii
C
bCAII
Vacuum i
5 s
Vacuum ii
D
PBS
PBS
Waste
Vacuum i
300 ms
Vacuum ii
E
Air
Air
Waste
300 ms
Vacuum ii
G
Waste
Vacuum i
4.8 s
Vacuum ii
H
bCAII
Vacuum i
300 ms
Vacuum ii
I
Air
1 s
PTFE tubing
J
PBS
500 ms
Waste
K
Air
500 ms
Waste
M
Air
500 ms
PTFE tubing
N
PBS
1 s
PTFE tubing

Fig. S1

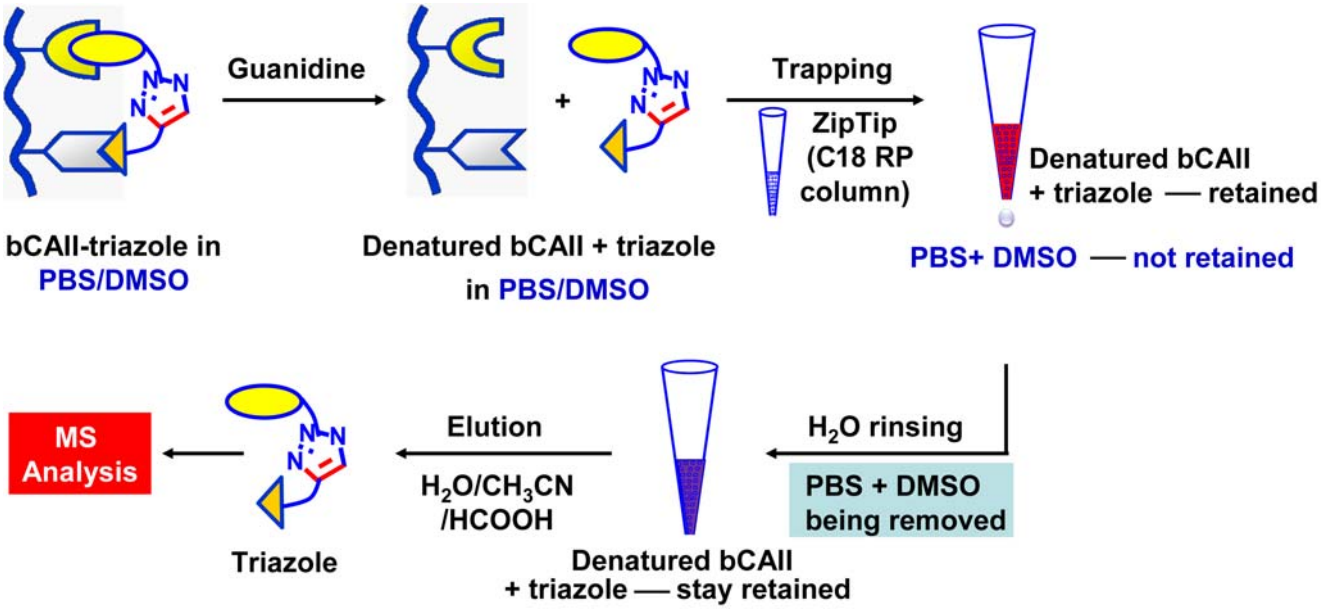

Guanidine
Trapping
ZipTip
(C18 RP
column)
Denatured bCAII
+ triazole — retained
bCAII-triazole in
PBS/DMSO
Denatured bCAII + triazole
in PBS/DMSO
PBS+ DMSO — not retained
MS
Analysis
Elution
H2O/CH3CN
/HCOOH
H2O rinsing
PBS + DMSO
being removed
Triazole
Denatured bCAII
+ triazole — stay retained

Fig. S2

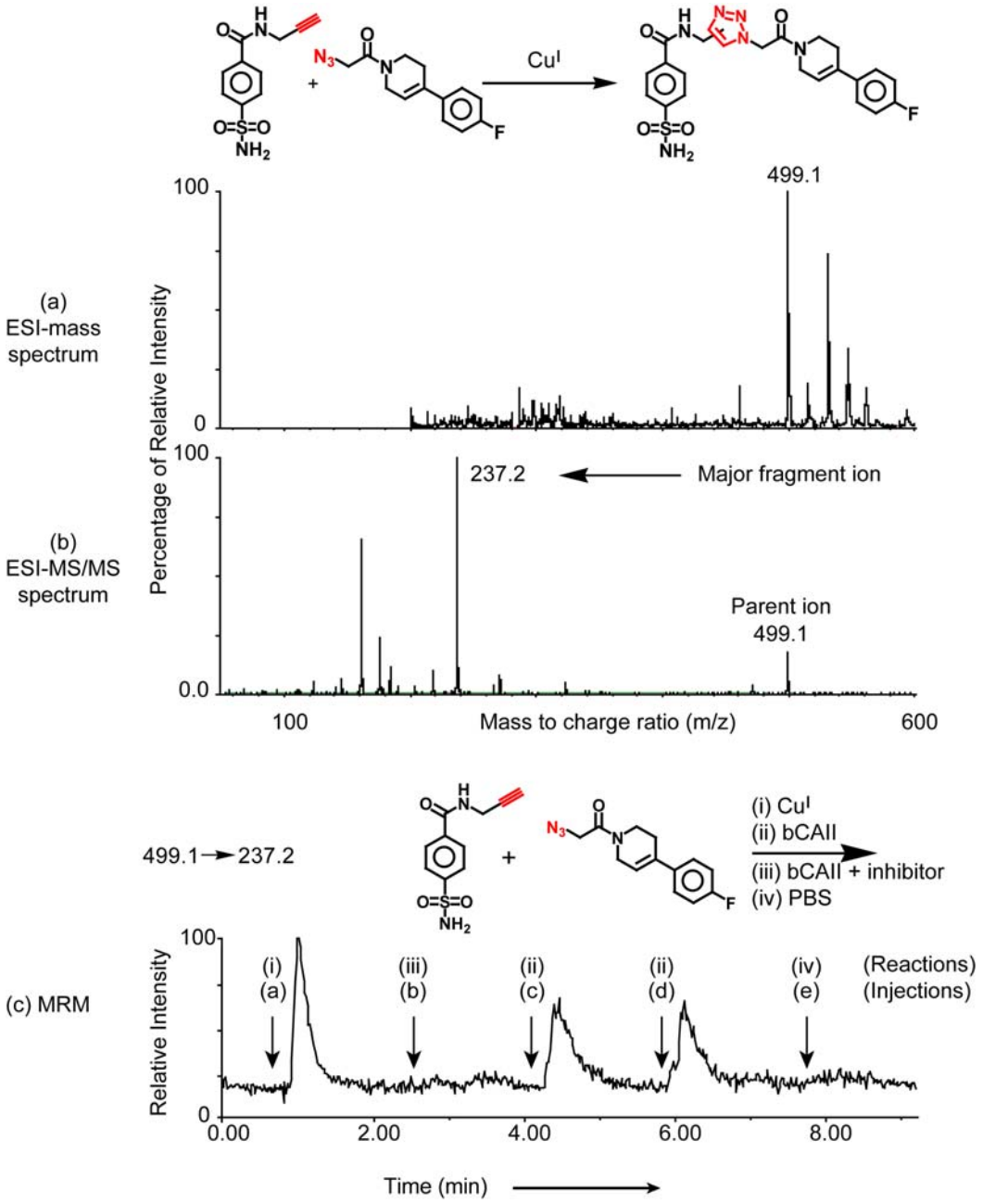

Cu^{I}
(a)
ESI-mass
spectrum
499.1
100
0
Percentage of Relative Intensity
(b)
ESI-MS/MS
spectrum
237.2
Major fragment ion
Parent ion
499.1
0.0
100
Mass to charge ratio (m/z)
600
499.1→237.2
(i) Cu^{I}
(ii) bCAII
(iii) bCAII + inhibitor
(iv) PBS
(c) MRM
Relative Intensity
(i) (a)
(iii) (b)
(ii) (c)
(ii) (d)
(iv) (e)
(Reactions)
(Injections)
0.00
2.00
4.00
6.00
8.00
Time (min)

Fig. S3

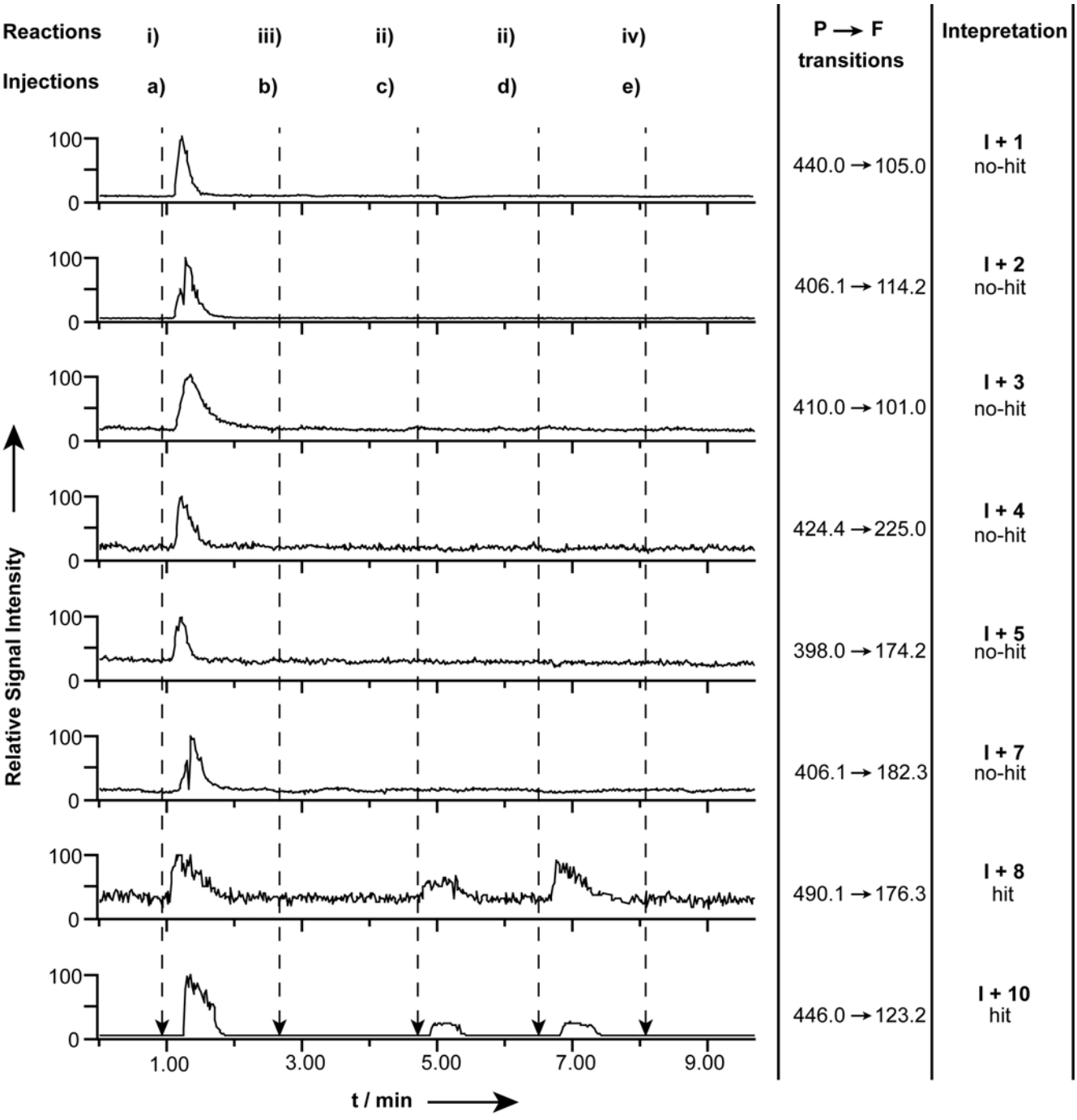

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
440.0 → 105.0
I + 1
no-hit
406.1 → 114.2
I + 2
no-hit
410.0 → 101.0
I + 3
no-hit
424.4 → 225.0
I + 4
no-hit
398.0 → 174.2
I + 5
no-hit
406.1 → 182.3
I + 7
no-hit
490.1 → 176.3
I + 8
hit
446.0 → 123.2
I + 10
hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (a)

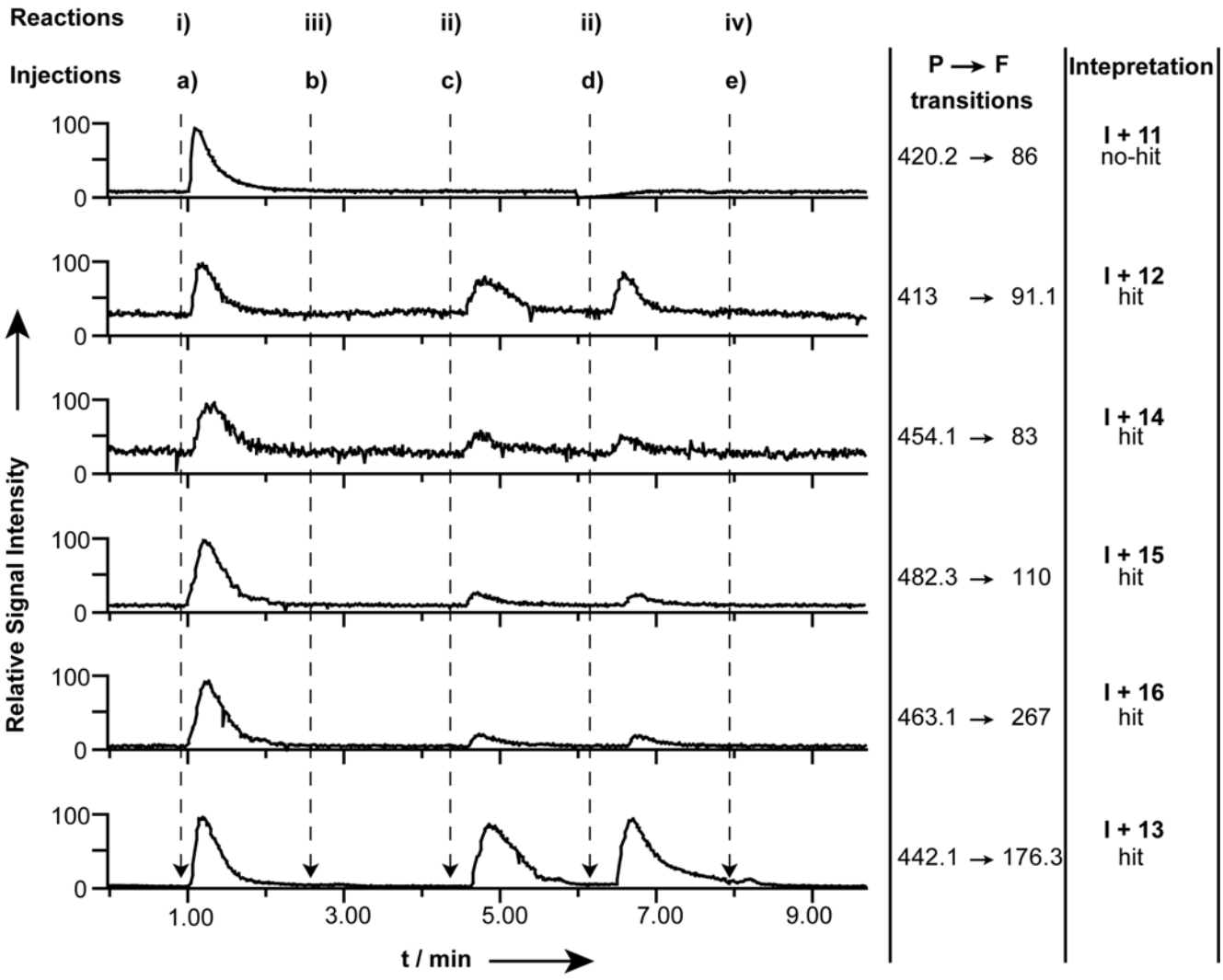

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
420.2 → 86
I + 11
no-hit
413 → 91.1
I + 12
hit
454.1 → 83
I + 14
hit
482.3 → 110
I + 15
hit
463.1 → 267
I + 16
hit
442.1 → 176.3
I + 13
hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (b)

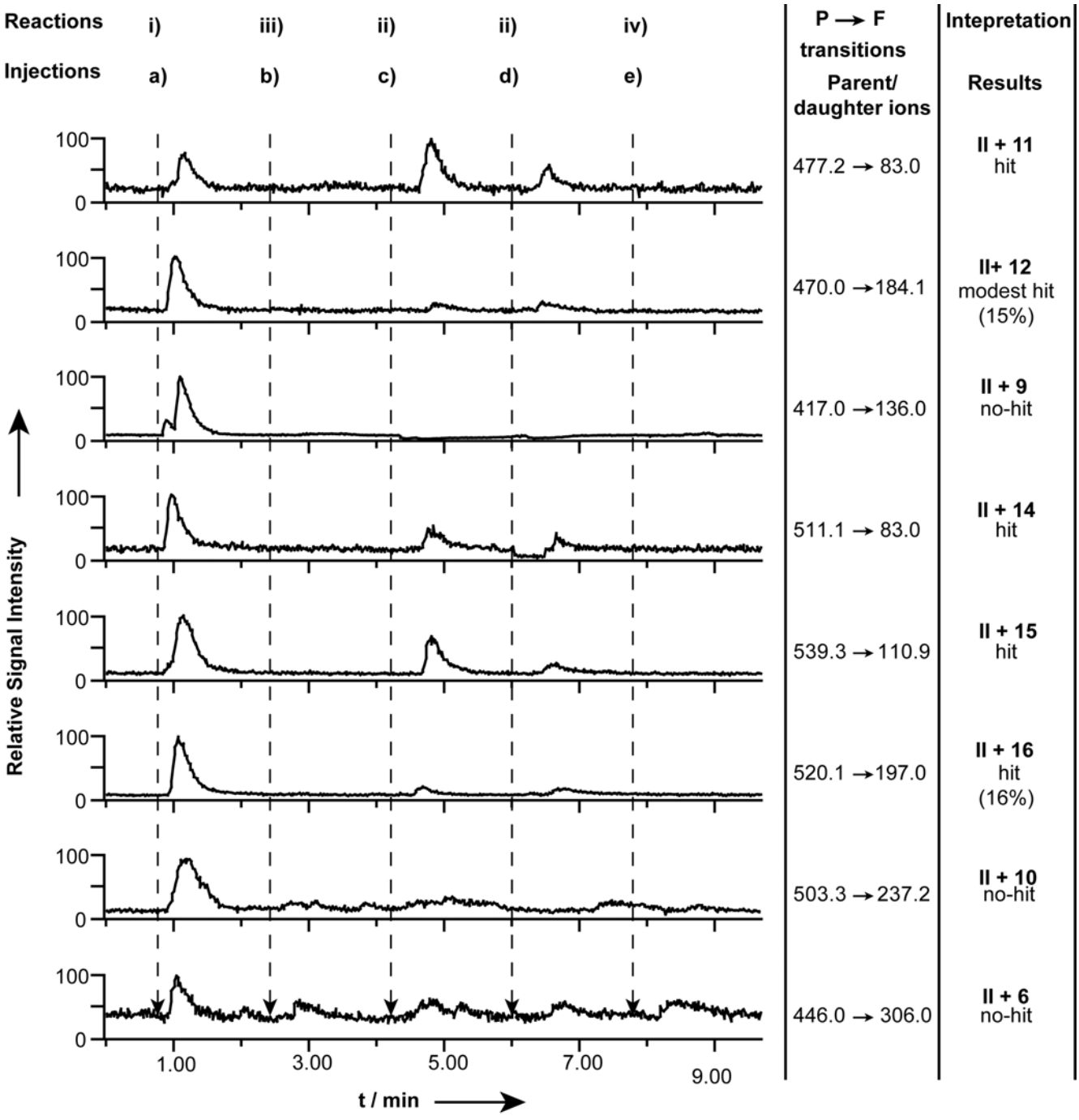

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Parent/ daughter ions
Intepretation
Results
477.2 → 83.0
II + 11
hit
470.0 →184.1
II+ 12
modest hit
(15%)
417.0 →136.0
II + 9
no-hit
511.1 → 83.0
II + 14
hit
539.3 → 110.9
II + 15
hit
520.1 →197.0
II + 16
hit
(16%)
503.3 → 237.2
II + 10
no-hit
446.0 → 306.0
II + 6
no-hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (c)

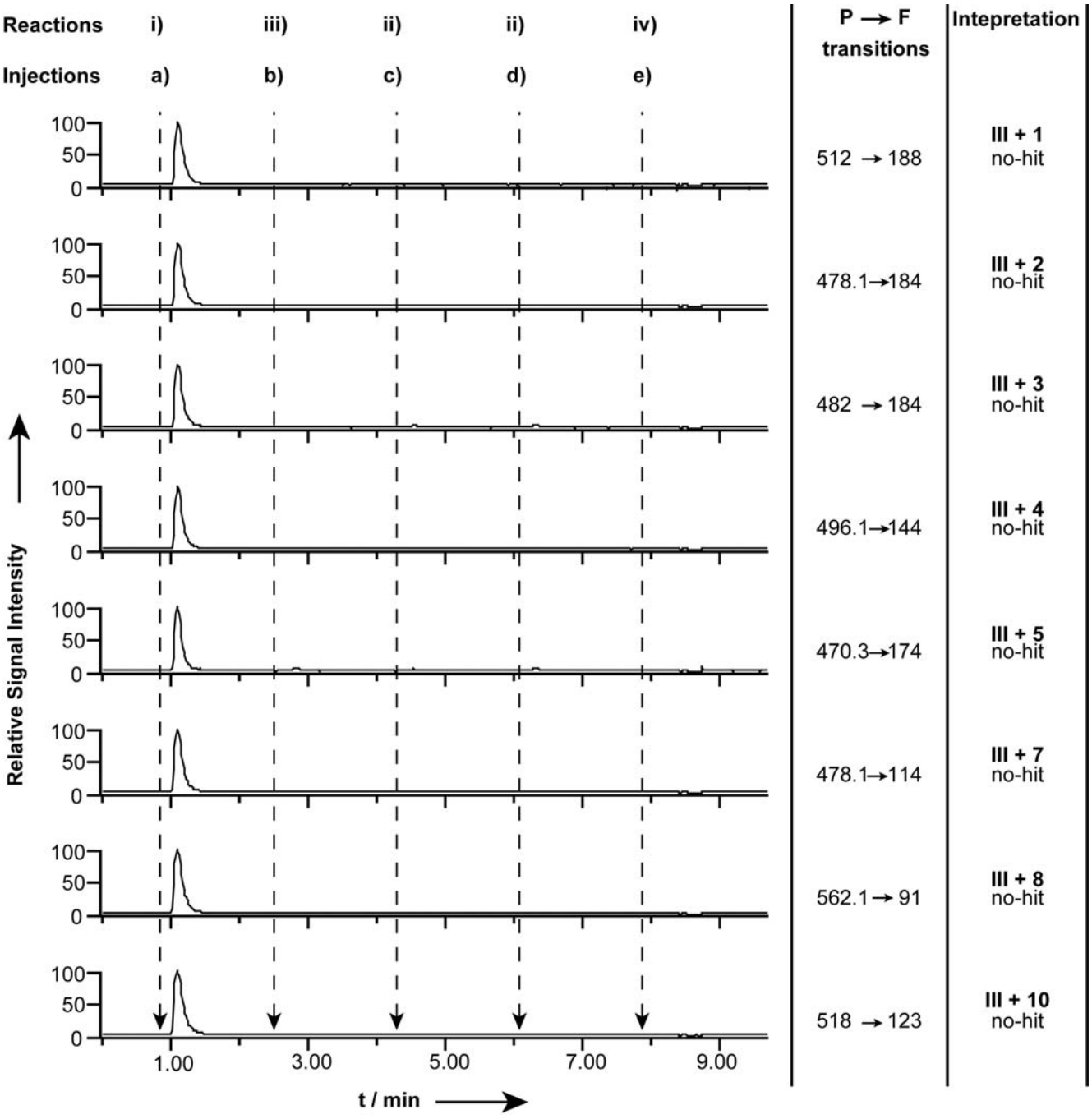

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
512 → 188
III + 1
no-hit
478.1→184
III + 2
no-hit
482 → 184
III + 3
no-hit
496.1→144
III + 4
no-hit
470.3→174
III + 5
no-hit
478.1→114
III + 7
no-hit
562.1→ 91
III + 8
no-hit
518 → 123
III + 10
no-hit
Relative Signal Intensity
100
50
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (d)

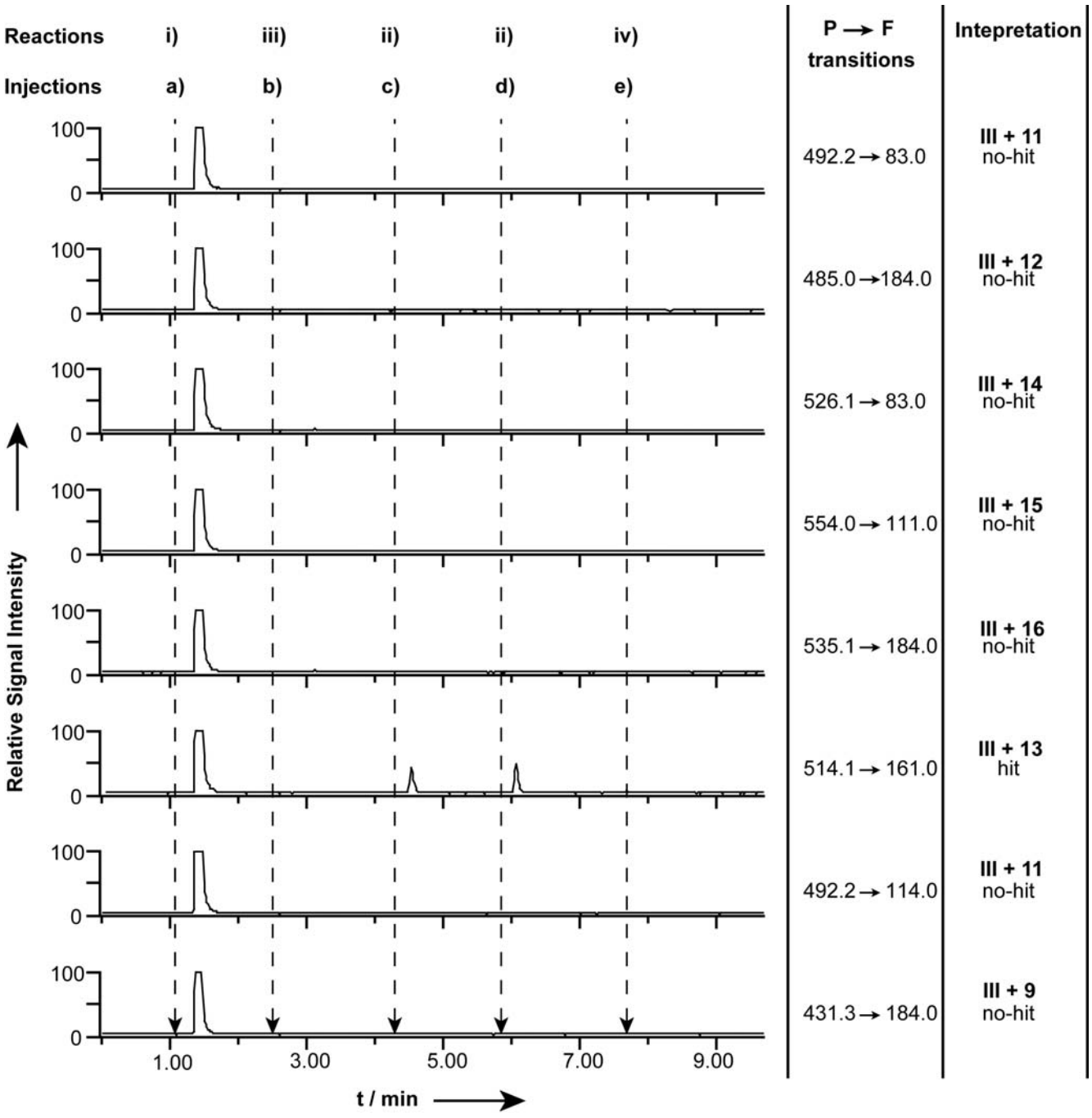

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
492.2 → 83.0
III + 11
no-hit
485.0 → 184.0
III + 12
no-hit
526.1 → 83.0
III + 14
no-hit
554.0 → 111.0
III + 15
no-hit
535.1 → 184.0
III + 16
no-hit
514.1 → 161.0
III + 13
hit
492.2 → 114.0
III + 11
no-hit
431.3 → 184.0
III + 9
no-hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (e)

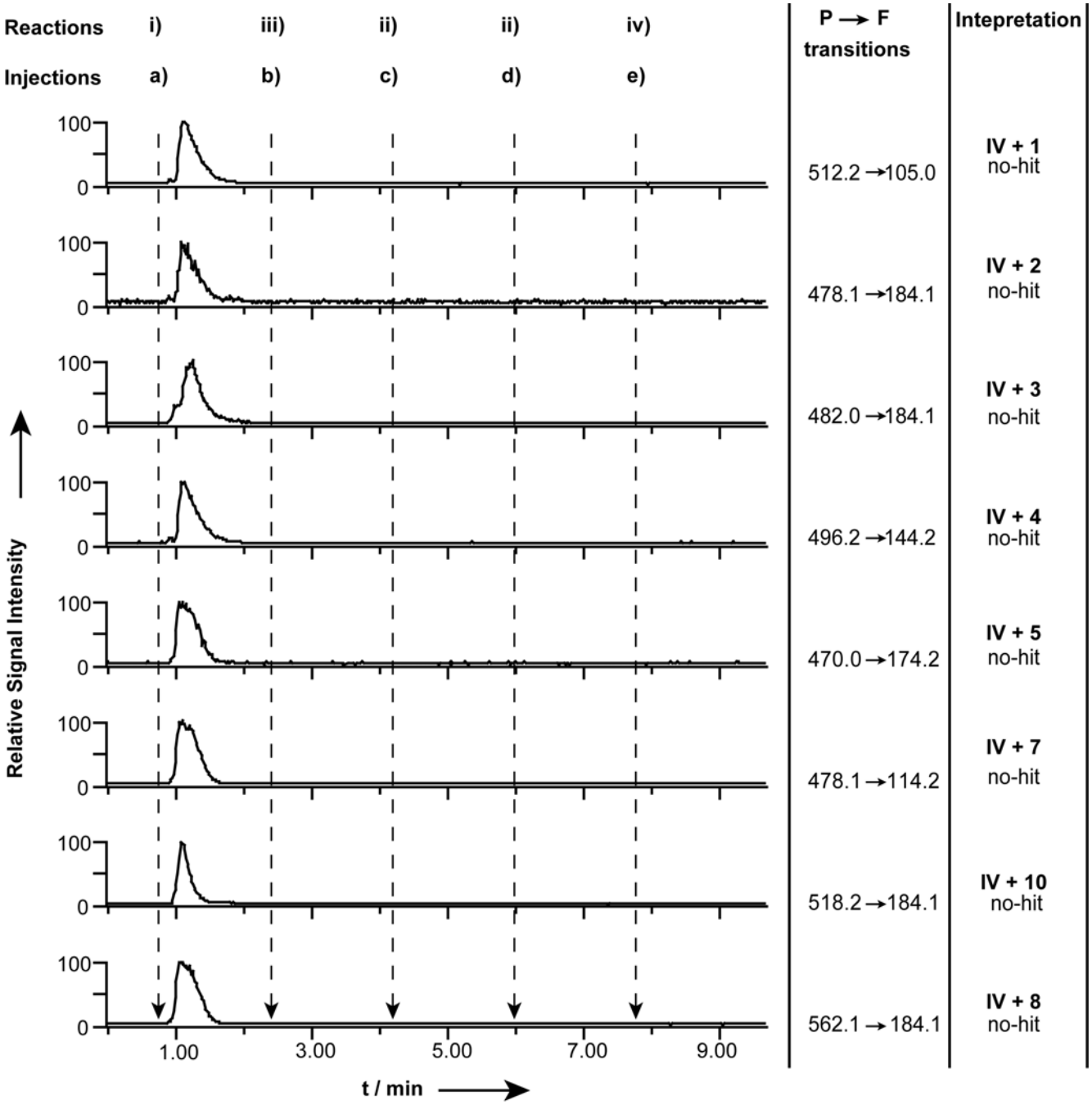

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
Relative Signal Intensity
100
0
512.2 →105.0
IV + 1
no-hit
478.1 →184.1
IV + 2
no-hit
482.0 →184.1
IV + 3
no-hit
496.2 →144.2
IV + 4
no-hit
470.0 →174.2
IV + 5
no-hit
478.1 →114.2
IV + 7
no-hit
518.2 →184.1
IV + 10
no-hit
562.1 → 184.1
IV + 8
no-hit
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (f)

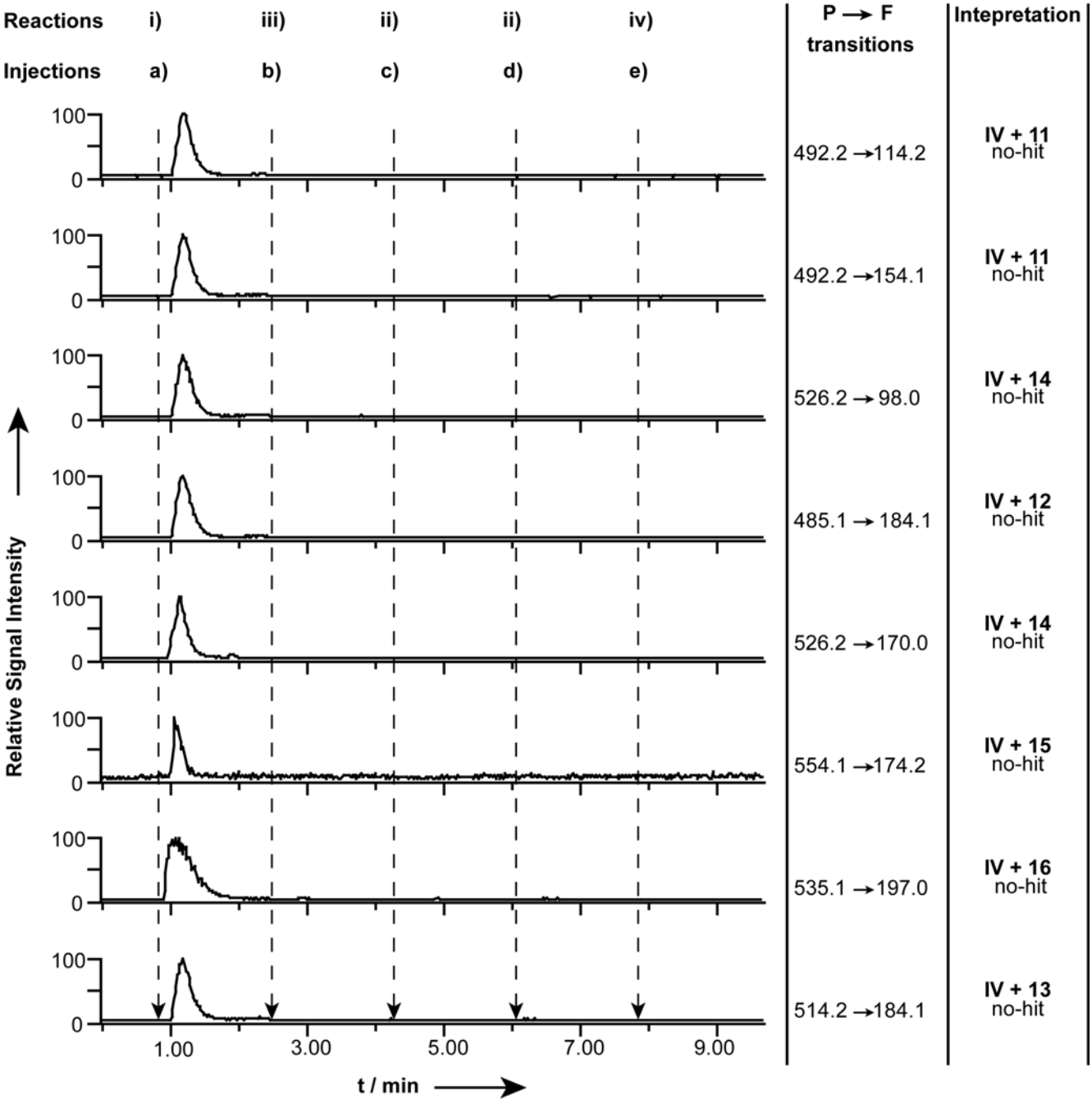

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
492.2 →114.2
IV + 11
no-hit
492.2 →154.1
IV + 11
no-hit
526.2 → 98.0
IV + 14
no-hit
485.1 → 184.1
IV + 12
no-hit
526.2 →170.0
IV + 14
no-hit
554.1 →174.2
IV + 15
no-hit
535.1 →197.0
IV + 16
no-hit
514.2 →184.1
IV + 13
no-hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (g)

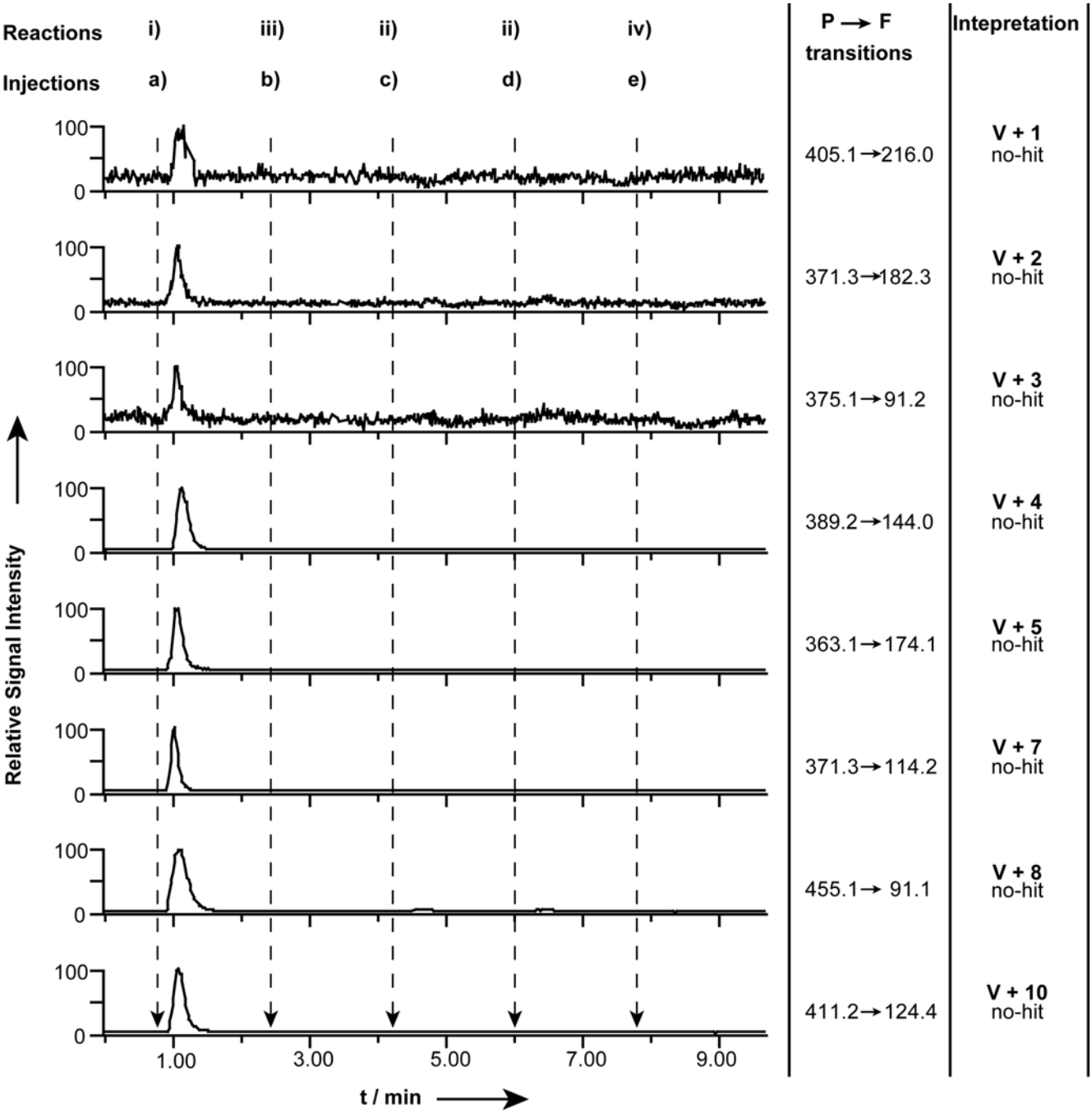

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
405.1→216.0
V + 1
no-hit
371.3→182.3
V + 2
no-hit
375.1→91.2
V + 3
no-hit
389.2→144.0
V + 4
no-hit
363.1→174.1
V + 5
no-hit
371.3→114.2
V + 7
no-hit
455.1→ 91.1
V + 8
no-hit
411.2→124.4
V + 10
no-hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (h)

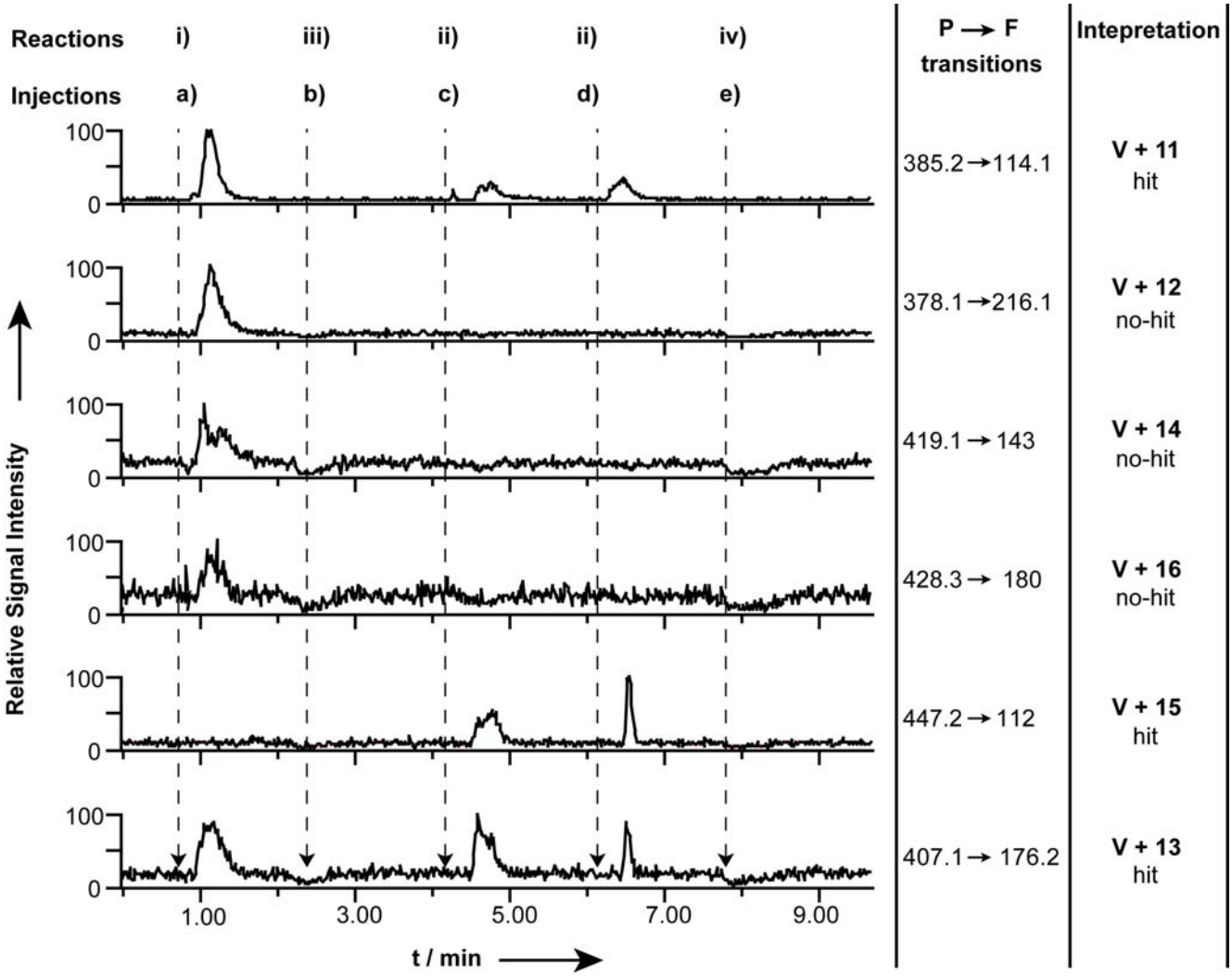

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
385.2→114.1
V + 11
hit
378.1→216.1
V + 12
no-hit
419.1→143
V + 14
no-hit
428.3→180
V + 16
no-hit
447.2→112
V + 15
hit
407.1→176.2
V + 13
hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (i)

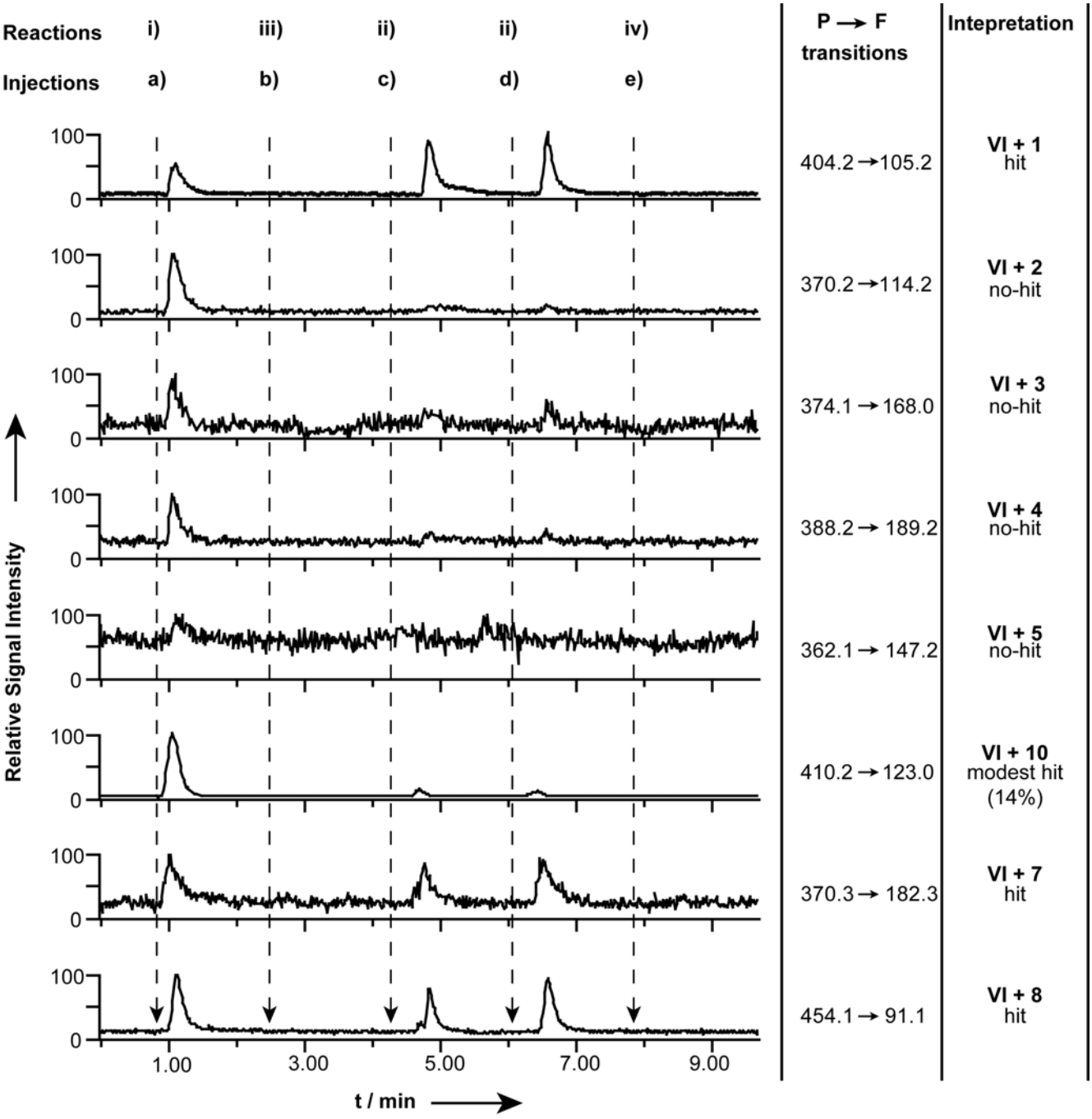

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
404.2 → 105.2
VI + 1
hit
370.2 → 114.2
VI + 2
no-hit
374.1 → 168.0
VI + 3
no-hit
388.2 → 189.2
VI + 4
no-hit
362.1 → 147.2
VI + 5
no-hit
410.2 → 123.0
VI + 10
modest hit
(14%)
370.3 → 182.3
VI + 7
hit
454.1 → 91.1
VI + 8
hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (j)

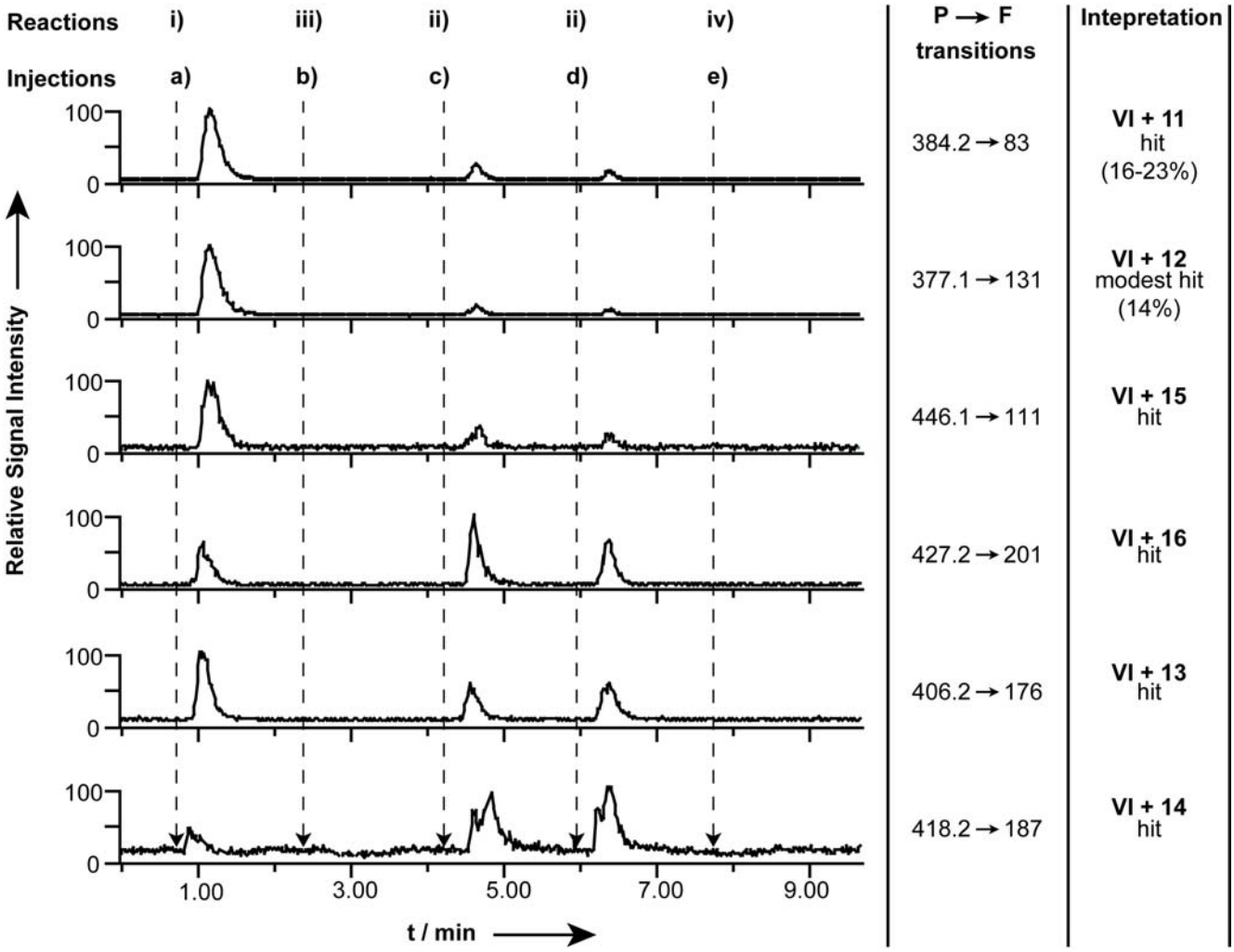

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
Relative Signal Intensity
100
0
384.2 → 83
VI + 11 hit (16-23%)
377.1 → 131
VI + 12 modest hit (14%)
446.1 → 111
VI + 15 hit
427.2 → 201
VI + 16 hit
406.2 → 176
VI + 13 hit
418.2 → 187
VI + 14 hit
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (k)

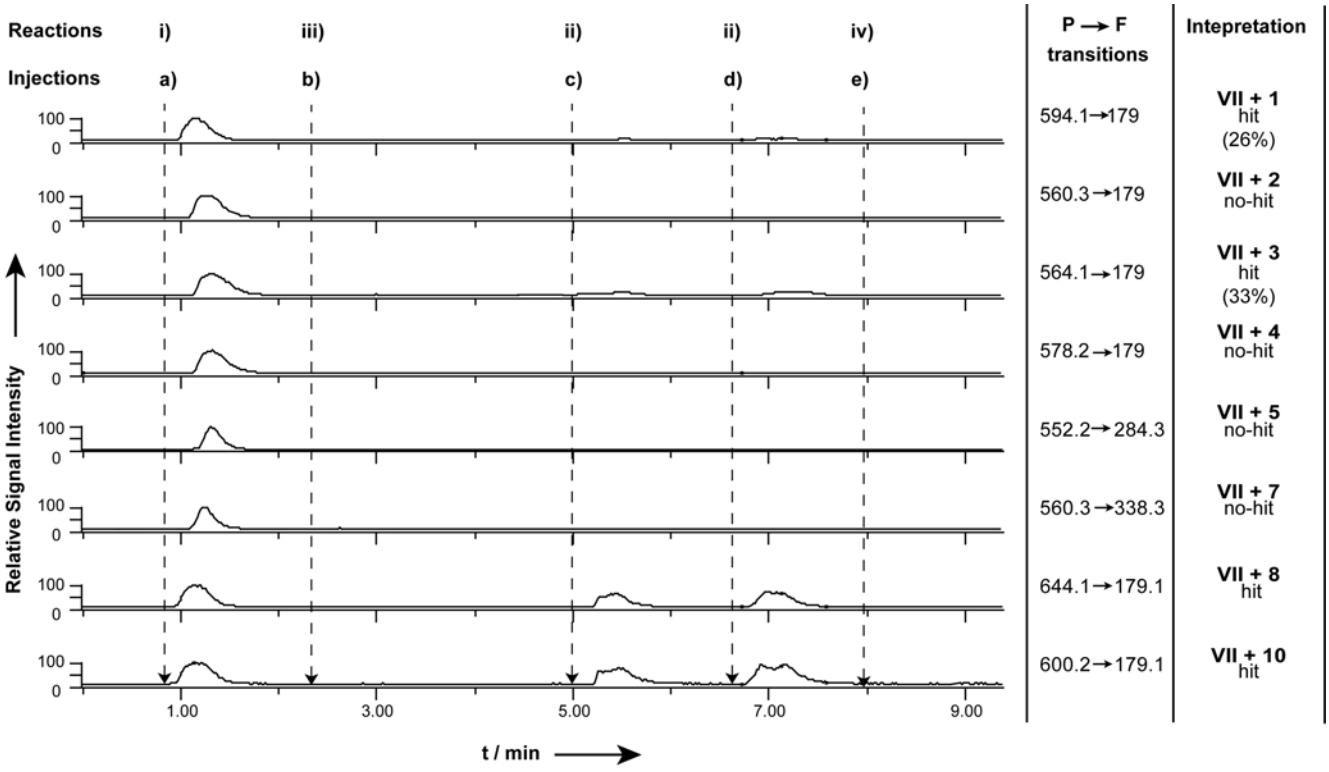


Fig. S4 (l)

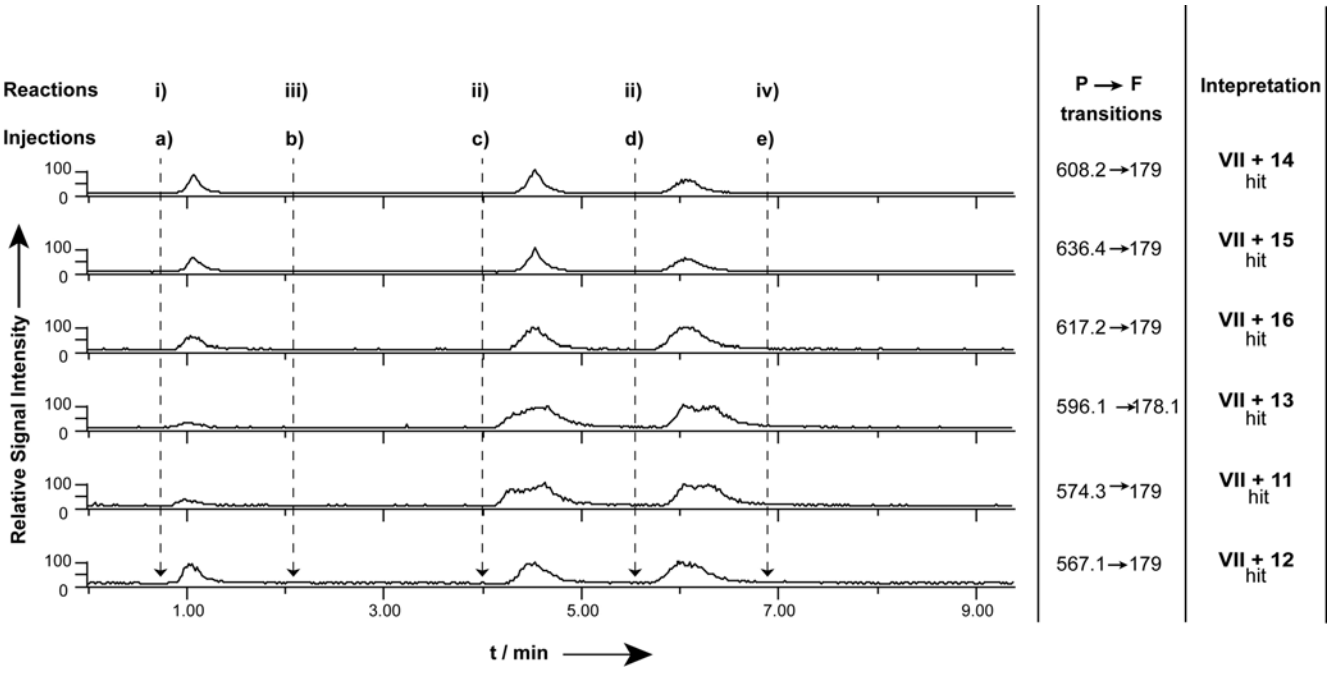


Fig. S4 (m)

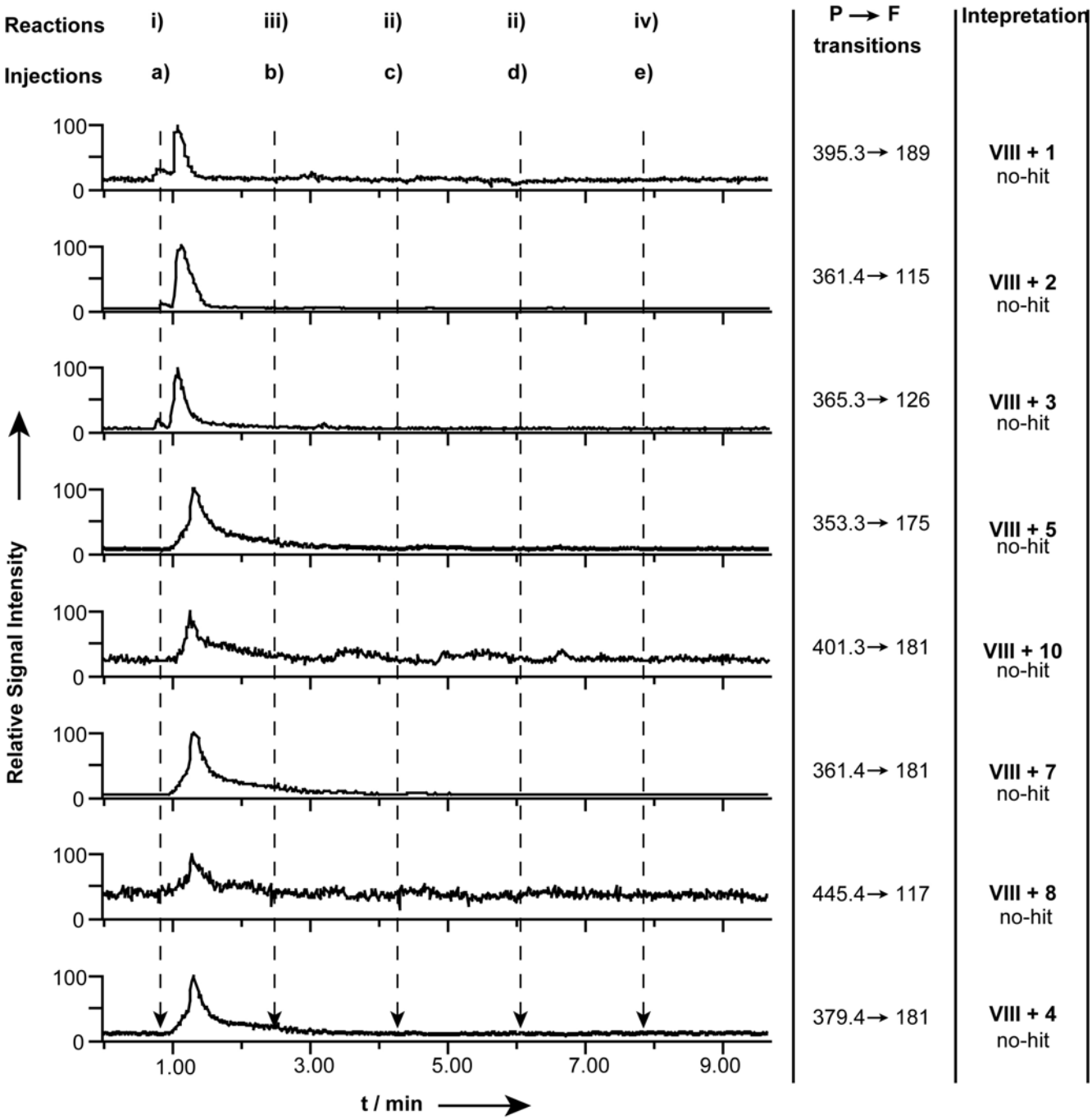

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
395.3→ 189
VIII + 1
no-hit
361.4→ 115
VIII + 2
no-hit
365.3→ 126
VIII + 3
no-hit
353.3→ 175
VIII + 5
no-hit
401.3→ 181
VIII + 10
no-hit
361.4→ 181
VIII + 7
no-hit
445.4→ 117
VIII + 8
no-hit
379.4→ 181
VIII + 4
no-hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S4 (n)

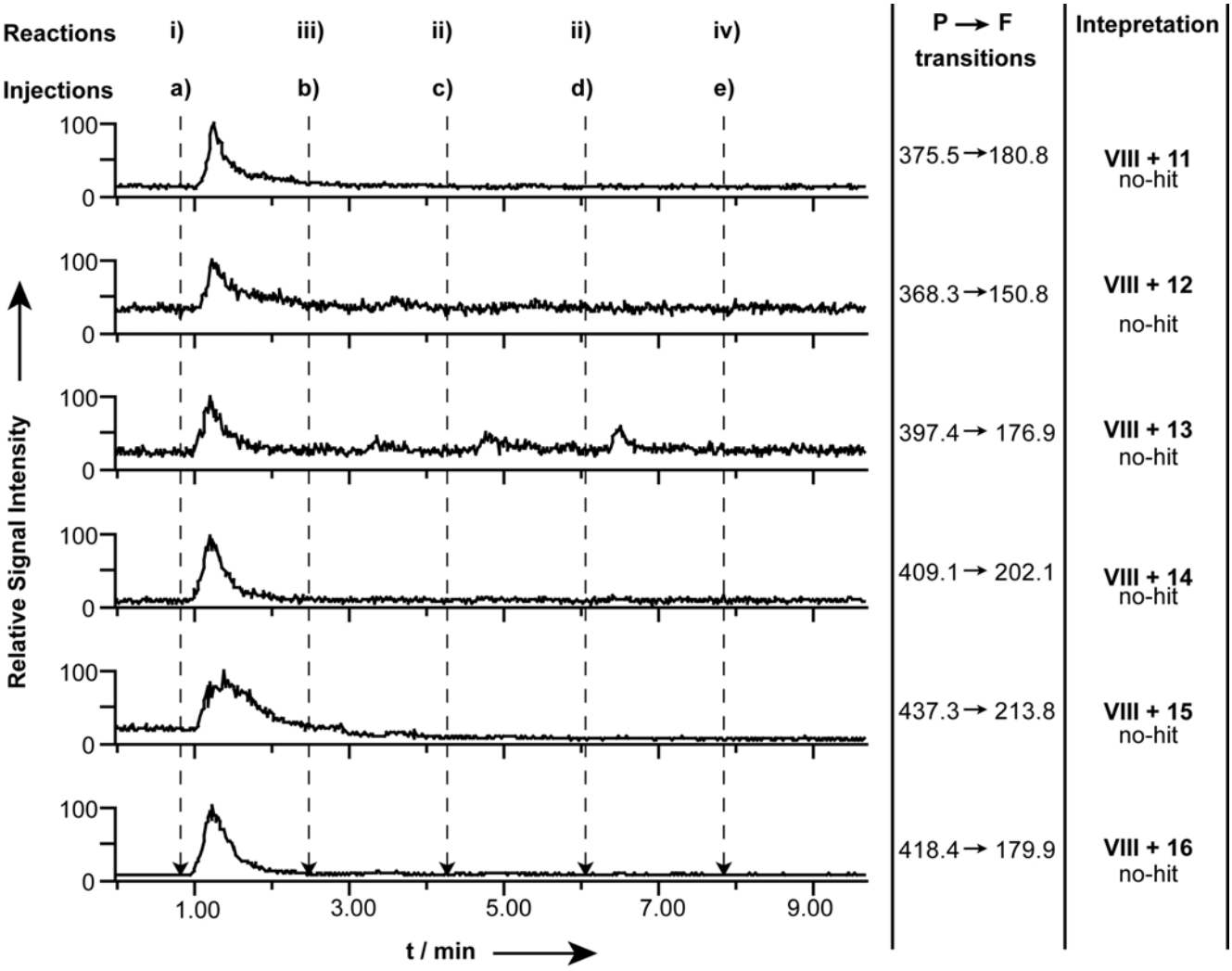
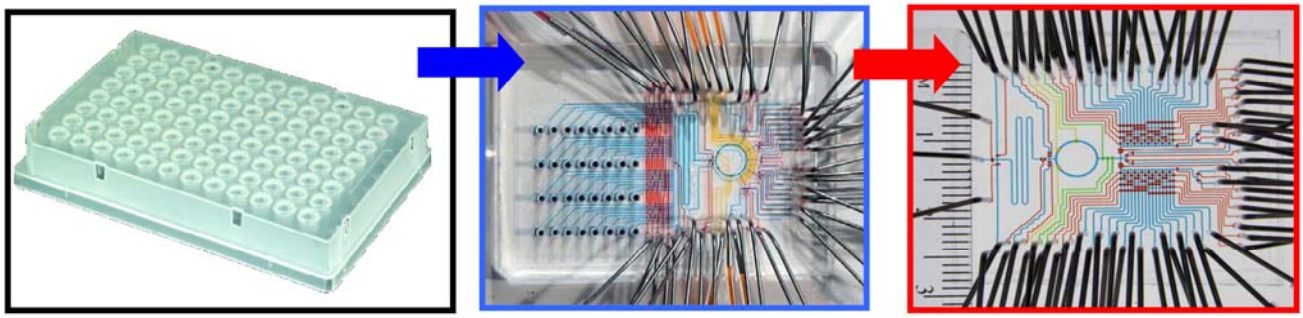

Reactions
i)
iii)
ii)
ii)
iv)
Injections
a)
b)
c)
d)
e)
P → F transitions
Intepretation
375.5→180.8
VIII + 11
no-hit
368.3→150.8
VIII + 12
no-hit
397.4→ 176.9
VIII + 13
no-hit
409.1→ 202.1
VIII + 14
no-hit
437.3→ 213.8
VIII + 15
no-hit
418.4→ 179.9
VIII + 16
no-hit
Relative Signal Intensity
100
0
1.00
3.00
5.00
7.00
9.00
t / min

Fig. S 4 (o)

Miniaturization of *in situ* Click Chemistry Reactions



Per cycle	96 Wells	1st-Generation	2nd-Generation
# of reaction	96	32	1024
Enzyme (bCAII)	94 μg	19 μg	0.36 μg
Acetylene	6 nmol	2.4 nmol	0. 12 nmol
Azide	40 nmol	3.6 nmol	0.12 nmol
Total reaction volume	100 μL	4 μL	0.4 μ L
Sample preparation time	Few mins	58 sec	15 sec
Hit identification time	40 mins	40 mins	15 sec
Detection method	LC-MS	LC-MS	MS/MS

Table S1