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**The phosphoinositide-specific phospholipase C inhibitor U73122 spontaneously forms conjugates with common components of cell culture medium**

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Running title

**U73122 chemical reactivity**

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Total pages 14

Number of tables 0

Number of figures 4

Number of references 24

Abstract word count 218

Introduction word count 314

Discussion word count 933

Abbreviations: PLC, phosphoinositide-specific phospholipase C; IP<sub>3</sub>, inositol 1,4,5-triphosphate; DTT, dithiothreitol; NEM, N-ethylmaleimide; HBSS, Hank's buffered saline solution; PBS, phosphate buffered saline; GSH, glutathione; DMEM, Dulbecco's Modified Eagle's medium without phenol red and L-glutamine; [Ca<sup>2+</sup>]<sub>i</sub> intracellular free Ca<sup>2+</sup>; A431, human epithelial carcinoma cell-line; DTT, dithiothreitol; MCDB131, optimised basal nutrient medium; EGF, epidermal growth factor, EGF; GFR, growth factor receptor; EGFR, epidermal GFR; FGFR, fibroblast GFR; PDGFR, platelet derived GFR; VEGFR, vascular endothelial GFR; c-Met, hepatocyte GFR.

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## Abstract

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in the regulation of  $\text{Ca}^{2+}$  release from inositol 1,4,5-triphosphate sensitive stores. U73122 (1-(6-((17 $\beta$ -3-methoxyestr-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) has been extensively used as a pharmacological inhibitor of PLC to elucidate the importance of this enzyme family in signal transduction pathways. U73122 has an electrophilic maleimide group, which readily reacts with nucleophiles such as thiols and amines. In the current study the conjugation of U73122 to common components of cell culture medium, namely L-glutamine, glutathione and bovine serum albumin (BSA) was demonstrated. The half-life of U73122 on incubation with phosphate buffered saline (PBS), Hank's buffered saline solution (with 2mM glutamine), optimised basal nutrient medium (MCDB131, without BSA), complete medium, Dulbecco's Modified Eagle's Medium (with 2mM L-glutamine) was ~150, 60, 32, 30 and 18 minutes respectively. However, U73122 was not recoverable from medium supplemented with 0.5% BSA. U73122 underwent hydrolysis of the maleimide group when incubated with PBS. Glutamine conjugates of U73122 were identified in cell culture medium. Furthermore, the inhibition of epidermal growth factor stimulated  $\text{Ca}^{2+}$  release in a human epidermoid carcinoma cell-line (A431) by U73122 was substantially reduced by the presence of BSA in a time-dependent manner. In complex cellular assays, the availability of U73122 to inhibit PLC may be limited by its chemical reactivity and lead to the misinterpretation of results in pharmacological assays.

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Phosphoinositide-specific phospholipase (PLC) enzymes catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol, key regulators of cellular responses (Williams and Katan 1996).

Overexpression of PLC in tumours with high receptor activity results in increased intracellular free Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) and cell proliferation (Piccolo et al. 2002; Wells and Grandis 2003). PLC<sub>γ</sub> is a point of convergent signalling from multiple growth factor receptors including EGFR, FGFR, c-Met, PDGFR and VEGFR2 (Matsuda et al. 2001; Katan et al. 2003) and is a key player in integrin-mediated cell motility processes (Piccolo et al. 2002; Jones et al. 2005).

U73122 (1-(6-((17β-3-methoxyestr-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) is firmly established as the archetypal inhibitor of PLC. Recently, the lack of selectivity of PLC inhibition by U73122 has been raised: U73122 was shown to possess oestrogenic activity (Cenni and Picard 1999) and to inhibit a range of unrelated proteins including 5-lipoxygenase (Feisst et al. 2005), histamine H1 receptor (Hughes et al. 2000), adenosine A<sub>1</sub> receptor (Walker et al. 1998) and plasma membrane Ca<sup>2+</sup> channels (Berven and Barritt 1995; Pulcinelli et al. 1998). U73122 is also more potent at inhibiting the mobilisation of Ca<sup>2+</sup> and plasma membrane Ca<sup>2+</sup> channels compared to PLC inhibition as reported by several authors (Berven and Barritt 1995; Grierson and Meldolesi 1995; Wang 1996).

This study identifies the conjugates of U73122 formed on incubation with standard cell culture media. The inactive analogue of U73122, namely U73343, in which the maleimide is substituted with a non-reactive succinimide, does not form these conjugates. We also show that the presence of BSA in the medium of a Ca<sup>2+</sup> release assay abolishes the inhibitory potency of U73122. It is proposed that the availability of U73122 to interact with PLC in an intact cell system will be severely hampered through conjugation of the maleimide group to

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non-specific biological nucleophiles and therefore inhibitory activity observed in cell-based assay cannot be assumed to be via PLC.

## Materials and Methods

**Materials.** U73122 (figure 1A) and U73343 (figure 1B) were obtained from Calbiochem (Merck, Nottingham, UK). Compounds were prepared as 5mM solutions in DMSO and stored at -80°C until required. Standards were checked for degradation products by LCMS immediately before use. Glutathione (GSH), L-glutamine, BSA (lipid-free), DMSO, MCDB131 medium, DMEM (without L-glutamine and phenol red), HBSS, complete medium, EGF and phosphate buffered saline tablets (PBS) were obtained from Sigma-Aldrich (Poole, UK). Fisher Scientific (Loughborough, UK) supplied HPLC grade solvents.

**PLC $\gamma$  inhibition in vitro.** Inhibition of PLC $\gamma$  by U73122 in the presence or absence of dithiothreitol (DTT; 1mM) was assessed as previously described (Bembenek et al. 2003; Katan et al. 2003). Briefly, after addition of enzyme, the rate of tritiated PIP<sub>2</sub> hydrolysis was monitored in the presence or absence of U73122 (0.5-100 $\mu$ M) with or without DTT (1mM). Human PLC $\gamma$ <sub>2</sub> used for this assay was sourced from insect (Sf9) cells expressing full-length PLC $\gamma$ <sub>2</sub> containing a C-terminal His<sub>6</sub> tag. Experimental details for the expression of PLC $\gamma$ <sub>2</sub> protein and its subsequent purification have been described previously (Rodriguez et al. 2001).

**Ca<sup>2+</sup> release assay in A431 cells.** U73122 was incubated with A431 (ATCC via LGC Promochem, Middlesex, UK), an epidermoid carcinoma cell line over expressing EGFR (Bembenek et al. 2003; Katan et al. 2003). A431 cells cultured in DMEM containing 2mM glutamine were harvested using TrypLE Express (Invitrogen Corporation, Paisley, UK) and seeded at a density of 5x10<sup>4</sup> cells per well in 100 $\mu$ L in a sterile flat clear bottomed black walled 96 well microtitre plate (Corning B.V. Life Sciences, Netherlands). Cells were

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incubated in a humidified incubator (5% CO<sub>2</sub> in air, 37°C) for 24h prior to the experiment to allow cells to attach. Probenecid was prepared in 1M NaOH (2.5mM), diluted (1:1 v/v) with 20mM HEPES in HBSS and 200µL added to 10mL of Calcium 3 dye (Molecular Devices Corp, Berkshire, UK). Medium was removed from the wells and replaced with 50µL of Calcium 3 dye containing 2.5µM probenecid and incubated for 60 minutes. During the incubation time, HBSS (50µL) containing 10µM U73122, 10µM U73122 and 4mM glutamine, 10µM U73122 and 1% BSA (lipid-free) or 0.1% DMSO was added at 30, 45, 55 or 60 minutes. Following incubation, cells were challenged with EGF (50µL of 5µg.ml<sup>-1</sup> in HBSS) and the Ca<sup>2+</sup> release response determined. Ca<sup>2+</sup> release was measured at 37°C on a FLIPR (Molecular Devices Corp., UK) with excitation 488nm and emission filter 510-570nm over a 2 minute period immediately after EGF addition.

**Incubation with cell culture medium.** U73122 or U73343 was incubated (37°C) with PBS (10mM), GSH (15 mg.ml<sup>-1</sup> in 10mM PBS), L-glutamine (2mM in HBSS), MCDB131 medium, MCDB131 supplemented with 0.5% BSA, complete medium, DMEM supplemented with 2mM L-glutamine. Samples were incubated directly on the LCMS autosampler (37°C) and aliquots (25µl) taken sequentially for analysis over a total incubation period of 60 minutes.

**Extraction of U73122 from plasma.** After addition of U73122 (1µM) to mouse plasma protein precipitation was effected by addition of 3 volume equivalents of either methanol or acetonitrile to the plasma sample. Following centrifugation (13000 x g, 5min, 4°C) the supernatant was analysed by LCMS. Liquid-liquid extraction using 10 volume equivalents of either ethyl acetate or dichloromethane was also undertaken, followed by centrifugation (1000 x g, 15min, 4°C) and evaporation of the organic layer to dryness under N<sub>2</sub>. Samples were reconstituted in mobile phase prior to analysis by LCMS. The impact of pH on extraction

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efficiency was also assessed by addition of an equal volume of either 0.1M HCl or 0.1M NaOH to the plasma sample prior to liquid-liquid extraction.

**LCMS analysis.** The Thermo Finnigan LC system consisted of a P4000 pump, AS3000 autosampler and SN4000 system controller interface (SpectraSystems, Thermo Separation Products, UK) connected to an iontrap MS (LCQ Classic, Finnigan) with Xcalibur data handling system (version 1.1). Chromatographic separation was facilitated on a Synergi 4 $\mu$  Polar-RP 80A 50x 3mm analytical column (Phenomenex, UK) using a mobile phase with initial conditions of 90% formic acid (0.1%) and 10% methanol at a total flow rate of 1ml.min<sup>-1</sup>. A linear gradient with the methanol content rising to 90% from 0.5 to 6min was initiated and held for a further 3.5min. Detection of analytes was achieved using the following LCQ conditions: N<sub>2</sub> sheath gas 80, N<sub>2</sub> auxiliary gas 20, spray voltage 4.5kV, capillary voltage 10V, capillary temperature 250°C and tube lens offset -10V. The collision energy for MS<sup>2</sup> and MS<sup>3</sup> analysis was set between 35 and 45V. Concentrations of the compound were normalised using the peak area of the compound analysed immediately following addition to the medium. Graphical representation of results was constructed using Prism (version 3.03, GraphPad Software Inc.).

## Results

**In vitro inhibition of the PLC $\gamma$  enzyme by U73122.** U73122 inhibited PLC $\gamma_2$  with an IC<sub>50</sub> value of 5.3 $\mu$ M. Further analysis found that in the presence of 1mM DTT, the inhibitory effect of U73122 was reduced substantially having an IC<sub>50</sub> value of >100 $\mu$ M (figure 1C). The inhibition of PLC by U73122 was in good agreement with other published data (Smith et al. 1990). The current assay was specifically designed for HTS implementation and the results obtained using U73122 confirmed its reliability and validity to identify PLC inhibitors in a HTS format.

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**U73122 covalently binds to glutathione, glutamine, BSA and plasma proteins.** U73122 was unstable in PBS having a half-life of ~150 minutes. The half-life of U73122 on incubation with HBSS containing 2mM glutamine, MCDB131 (without BSA), complete medium, and DMEM (with 2mM L-glutamine) was 60, 32, 30 and 18 minutes respectively. U73122 was not recoverable from medium supplemented with 0.5% BSA immediately after its addition indicating that the compound instantly bound to BSA substituents. Similarly, U73122 was not recoverable from mouse plasma regardless of the rigorous extraction methods employed (data not shown). In contrast, U73343 was stable on incubation with cell culture medium (figure 1D).

Two products were detected on incubation of U73122 with PBS, one of which had the expected  $[M+H]^+$  ion of U73122 ( $m/z$  465; figure 2A). The  $MS^2$  spectrum of U73122 had fragmentation ions of  $m/z$  173 ( $C_{12}H_{13}O^+$ ),  $m/z$  197 ( $C_{10}H_{17}N_2O_2^+$ ) and  $m/z$  269 ( $C_{19}H_{25}O^+$ ) (figure 3A). The second product had a  $[M+H]^+$  ion of  $m/z$  483 (18 amu greater than U73122; figure 2B). The  $MS^2$  spectrum of  $m/z$  483 showed hydrolysis occurred on the maleimide group (figure 3B). This phenomenon has been previously described for a maleimide functional group (Khan 1984).

On incubation of U73122 with glutathione, a ubiquitous nucleophile in biological systems, a  $[M+H]^+$  ion of  $m/z$  772 was detected corresponding to the glutathione conjugate of U73122 (figure 2C).  $MS^2$  analysis resulted in loss of 129Da, a common neutral loss observed for thiol-linked glutathione conjugates (Ma and Subramanian 2006).  $MS^3$  analysis (figure 3C) revealed a spectrum containing further product ions indicating a thiol-linked glutathione conjugate ( $m/z$  499:  $C_{29}H_{43}N_2O_3S^+$ ) and product ions characteristic of U73122 ( $m/z$  465 and  $m/z$  269).

From these results it was predicted that U73122 would undergo conjugation with thiol-containing compounds present in cell culture medium such as cysteine. However, U73122-



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cysteine conjugates were not detected following incubation of U73122 with culture medium. The predominant  $[M+H]^+$  ion detected after incubation of U73122 with cell culture medium had a  $m/z$  of 611 which corresponded to the U73122-glutamine conjugate (figure 2D).

Incubation of U73122 with L-glutamine alone resulted in the formation of a species with an  $[M+H]^+$  ion and retention time identical to that observed in the culture media.  $MS^2$  analysis of the molecular ion revealed a spectrum with 2 product ions of  $m/z$  465 and  $m/z$  594 (data not shown).  $MS^3$  analysis showed that the  $m/z$  465 ion gave the spectrum characteristic of U73122 itself confirming that the molecular ion was a conjugate of U73122 (data not shown). The  $m/z$  594 product ion of  $[M+H]^+$  ion 611 arose from loss of 17Da and showed further loss of 28Da. These neutral losses are likely to arise from successive losses of ammonia and CO from the amide group (figure 3D).

**Inhibition of EGF-stimulated  $Ca^{2+}$  release from A431 cells.** The inhibition of an EGF evoked release of  $Ca^{2+}$  by U73122 was measured in A431 cells and the effect of addition of glutamine and BSA on potency was assessed. U73122 readily formed a conjugate with glutamine and therefore it was anticipated that addition of glutamine would decrease the activity of the compound. However, glutamine did not appear to diminish the potency of U73122 inhibition of EGF stimulated  $Ca^{2+}$  release (data not shown). BSA (0.5%) caused a marked reduction in the potency of U73122 that was dependent on the pre-incubation time of the cells with the compound and BSA (figure 4A). After 30 minutes incubation of U73122 (5 $\mu$ M) and 0.5% BSA with the A431 cells no inhibition of EGF elicited  $Ca^{2+}$  release was detected and the  $Ca^{2+}$  curve was identical to that without addition of U73122.

## Discussion

U73122 is a weak inhibitor ( $IC_{50}$  5 $\mu$ M) of PLC in the enzyme-based assay. The reactivity of the maleimide group is critical for this activity as its replacement with a succinimide (U73343), or co-incubation with DTT, renders the compound inactive. However, the inherent

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reactivity of the maleimide functional group may prevent U73122 interacting effectively with PLC in more complex cell-based assays. In A431 cells, U73122 was capable of inhibiting the  $\text{Ca}^{2+}$  release response following stimulation with EGF. Although, inhibition of  $\text{Ca}^{2+}$  can be rationalised by PLC inhibition, there are no definitive data demonstrating that U73122 exerts this effect by directly interacting with PLC in a cellular assay. The supplementation of HBSS with 0.5% BSA caused a reduction in the inhibitory potency of U73122 even when pre-incubated for as little as 5 minutes with the cell line. This clearly demonstrates competition for U73122 between cellular proteins and BSA.

On incubation of U73122 with PBS the compound was chemically unstable and readily underwent hydrolysis of the maleimide group, a reaction which has been previously characterised maleimide itself (Khan 1984). U73122 readily formed a conjugate with glutathione, a ubiquitous thiol source present in cells. The spectral data obtained for the U73122-glutathione conjugate supports the proposition that conjugation occurs via the thiol of glutathione, showing neutral loss of 129Da and a fragment ion of U73122 conjugated to a thiol group on the maleimide (figure 3C). Similarly, the conjugation of N-ethylmaleimide and maleimidod sugars with the thiol of glutathione has been previously reported (Lee and Samuels 1961; Shin et al. 2001).

Surprisingly, U73122 did not appear to form thiol conjugates when incubated with cysteine-rich media MCDB131 or DMEM. Conjugation of U73122 with L-glutamine was identified as the principal component in cell culture medium and its formation was confirmed on incubation of U73122 with 2mM L-glutamine in PBS. Glutamine contains 3 nucleophilic centres that can undergo conjugation with the maleimide moiety: an amide, amino and carboxylic acid group. However spectral data excluded the possibility of conjugation through the amide group. It anticipated that the glutamate would react via the more nucleophilic

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amino group (as depicted in Figure 3D) rather than the carboxylate. Though this cannot be confirmed based on the mass spectral data obtained, maleimide conjugation to glutamine via the amino group has been reported previously (Sharpless and Flavin 1966). The lack of thiol conjugation in medium could either result from L-glutamine having more nucleophilic characteristics under the experimental conditions or the instability of thiol conjugates formed prevented their detection. Supplementation of cell culture medium with 0.5% BSA resulted in the instantaneous disappearance of U73122 probably by covalent binding to nucleophilic centres present on BSA. It was reported that maleimide analogues of the anticancer drugs doxorubicin and carboplatin selectively conjugated to the cysteine-34 position of human serum albumin (Warnecke et al. 2004) and therefore it is conceivable that U73122 conjugates to the equivalent cysteine residue in BSA. However conjugation of U73122 to other nucleophilic centres cannot be discounted. These data clearly indicate that although the maleimide is reactive and capable of reacting with a number of nucleophilic species, there was a level of selectivity probably governed by chemical and steric constraints.

The maleimide group undergoes a Michael addition reaction with biological nucleophiles to form the respective conjugate (figure 4B). The electron deficient double bond, activated by the two carbonyl groups, present in the maleimide of U73122, is absent in U73343 and therefore this type of reactivity is precluded for the inactive analogue. The chemical reactivity of U73122 must raise questions about the ability of the compound to distribute effectively within a cell-based assay (or *in vivo*) and interact with the target enzyme. Alkylation by the reactive maleimide moiety of U73122 has previously been suggested to account for the non-specific effects of U73122 (Horowitz et al. 2005). Whilst U73122 inhibited PLC-dependent processes in cells (measured by PIP<sub>2</sub> decrease/IP<sub>3</sub> increase and DAG production), it also had many non-PLC mediated effects that could be mimicked by N-ethylmaleimide (NEM) (Horowitz et al. 2005). Pre-treatment of cells with NEM prevented

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the additional effects of U73122 (or *vice versa*) indicating that NEM and U73122 were capable of alkylating the same nucleophilic groups (Horowitz et al. 2005).

In cell-based assays, therefore, U73122 will rapidly bind to exposed nucleophilic thiols and/ or amines either present in macromolecules in the medium (e.g. serum or albumin) or on plasma membrane proteins limiting the availability of the compound to interact with PLC in intact cells. In the current study, the presence of glutamine in the  $\text{Ca}^{2+}$  assay did not interfere with the inhibition of EGF-stimulated calcium release by U73122 even though it readily conjugated with glutamine. However, U73122 had a half-life of 60 minutes when incubated with glutamine in HBSS (figure 1D) thus it appears that a sufficient concentration remained to elicit an affect in the cell assay. This is in contrast to the addition of BSA to the medium where a rapid change in the potency of U73122 was observed demonstrating the reactivity to BSA nucleophilic centres. Therefore, the availability of U73122 in cell-based assays that incorporate supplementation of culture medium with BSA or serum will be extremely short-lived. Caution is recommended in using U73122 to attribute PLC-dependent cellular events, as the inherent chemical reactivity of U73122 will have an impact on the experimental outcome in the presence of biological nucleophiles (i.e. any cell based assay). Therefore, it is vitally important for the advancement of understanding the role of PLC in signal transduction pathways to identify a chemically stable PLC inhibitor to replace U73122, and/or to supplement the studies with inhibition of gene or protein expression (e.g. antisense oligonucleotides, siRNA).

### **Acknowledgements**

We thank Sam Ranasinghe, University College London, for technical support.

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## Footnotes

This work was supported by Cancer Research UK [CUK] programme grant number C309/A8274.

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## Figure Legends

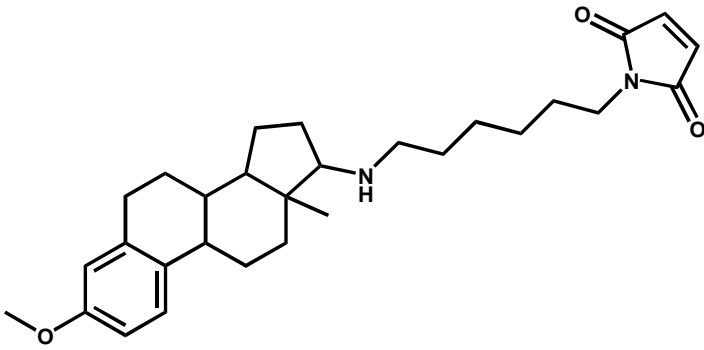
Figure 1. (A) U73122, (1-[6-((17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione); (B) the inactive succinimide containing analogue U73343 1-[6-((17 $\beta$ -3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione; (C) Inhibition (IC<sub>50</sub>) of PLC $\gamma$  by U73122 determined using Phospholipid FlashPlates™, in the absence ( $\blacktriangle$ ;  $r^2$  0.98 Chi, Hillslope 2.6) and presence ( $\blacksquare$ ;  $r^2$  0.67 Chi, Hillslope 1.3) of DTT (1mM) and (D) Disappearance of U73122 (5 $\mu$ M) after incubation (37°C) with MCDB131 without BSA ( $\blacksquare$ ), DMEM supplemented with 2mM L-glutamine ( $\blacktriangle$ ), complete medium ( $\blacktriangledown$ ), HBSS containing 2mM glutamine ( $\blacksquare$ ) or PBS ( $\triangle$ ) and stability of U73343 (5 $\mu$ M) after incubation (37°C) with MCDB131 without BSA ( $\square$ ).

Figure 2. Extracted ion chromatogram of the [M+H]<sup>+</sup> ion of (A) U73122 (m/z 465), (B) U73122 hydrolysis product (m/z 483), (C) U73122-glutathione conjugate (m/z 772) and (D) U73122-glutamine conjugate (m/z 611).

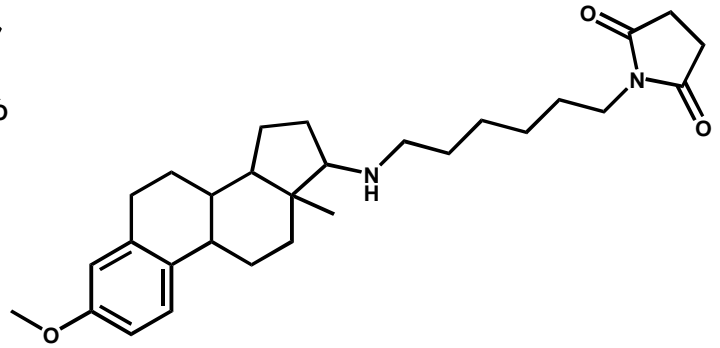
Figure 3. Spectra and proposed fragmentation patterns for (A) MS<sup>2</sup> of U73122, (B) MS<sup>2</sup> of U73122 hydrolysis product, (C) MS<sup>3</sup> of U73122-glutathione product and (D) MS<sup>3</sup> U73122-glutamine product.

Figure 4. (A) Inhibition of EGF-stimulated Ca<sup>2+</sup> release in A431 cells after co-incubation of the cells with 5 $\mu$ M U73122 and 0.5% BSA for 0, 5, 15 and 30 minutes (n=3) and (B) Michael addition reaction of the conjugation of the maleimide of U73122 with biological nucleophiles (<sup>o</sup>Nuc).

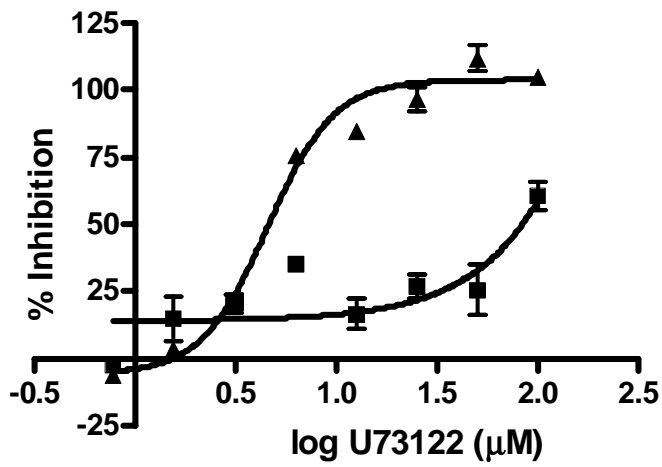
**A**



**B**



**C**



**D**

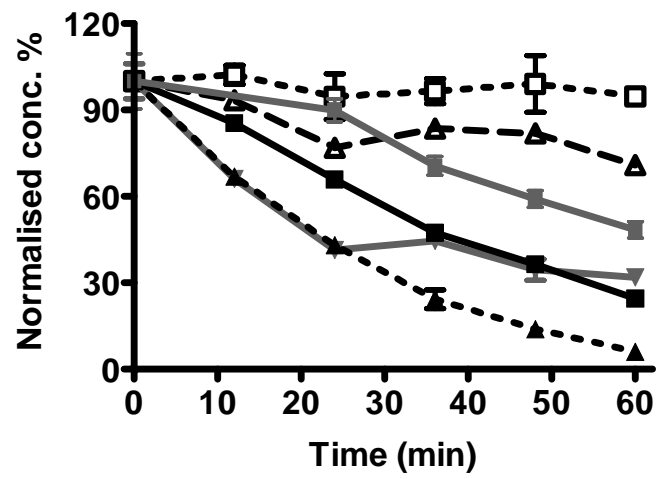


FIGURE 1

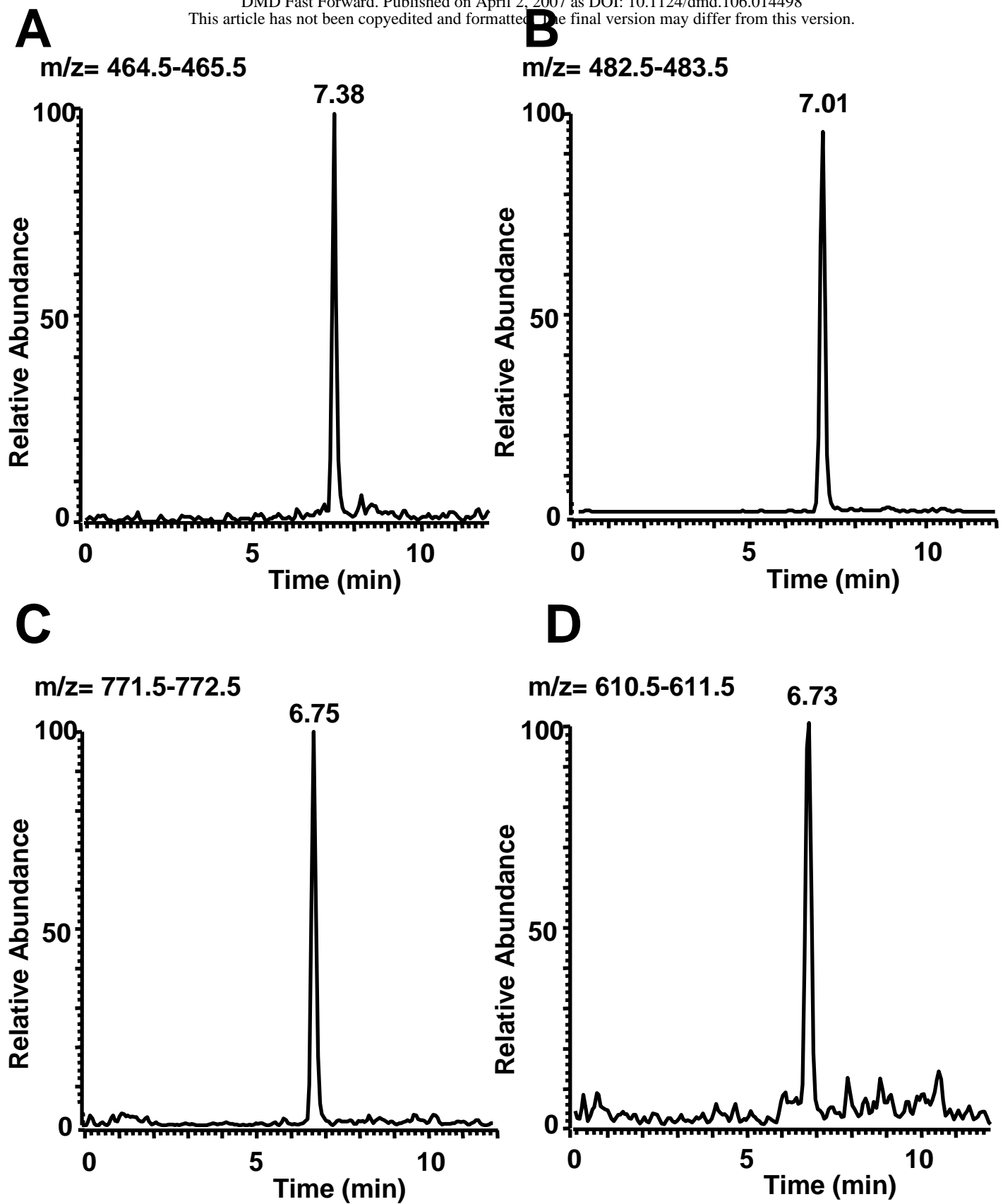


FIGURE 2

# A

F: + c ESI Full ms2 465@45 [130-500]

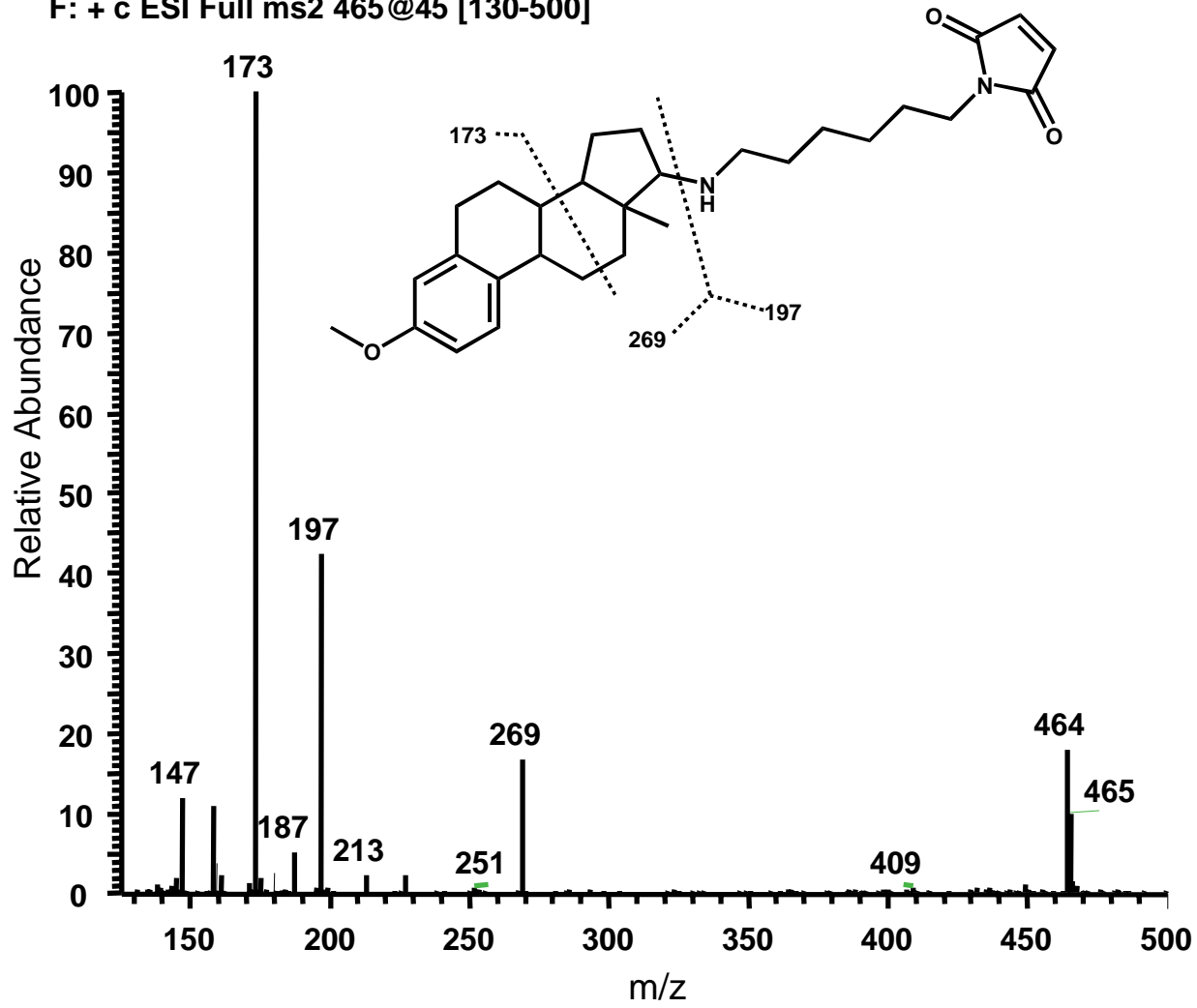


FIGURE 3A

# B

F: + c ESI Full ms2 483@45 [130-500]

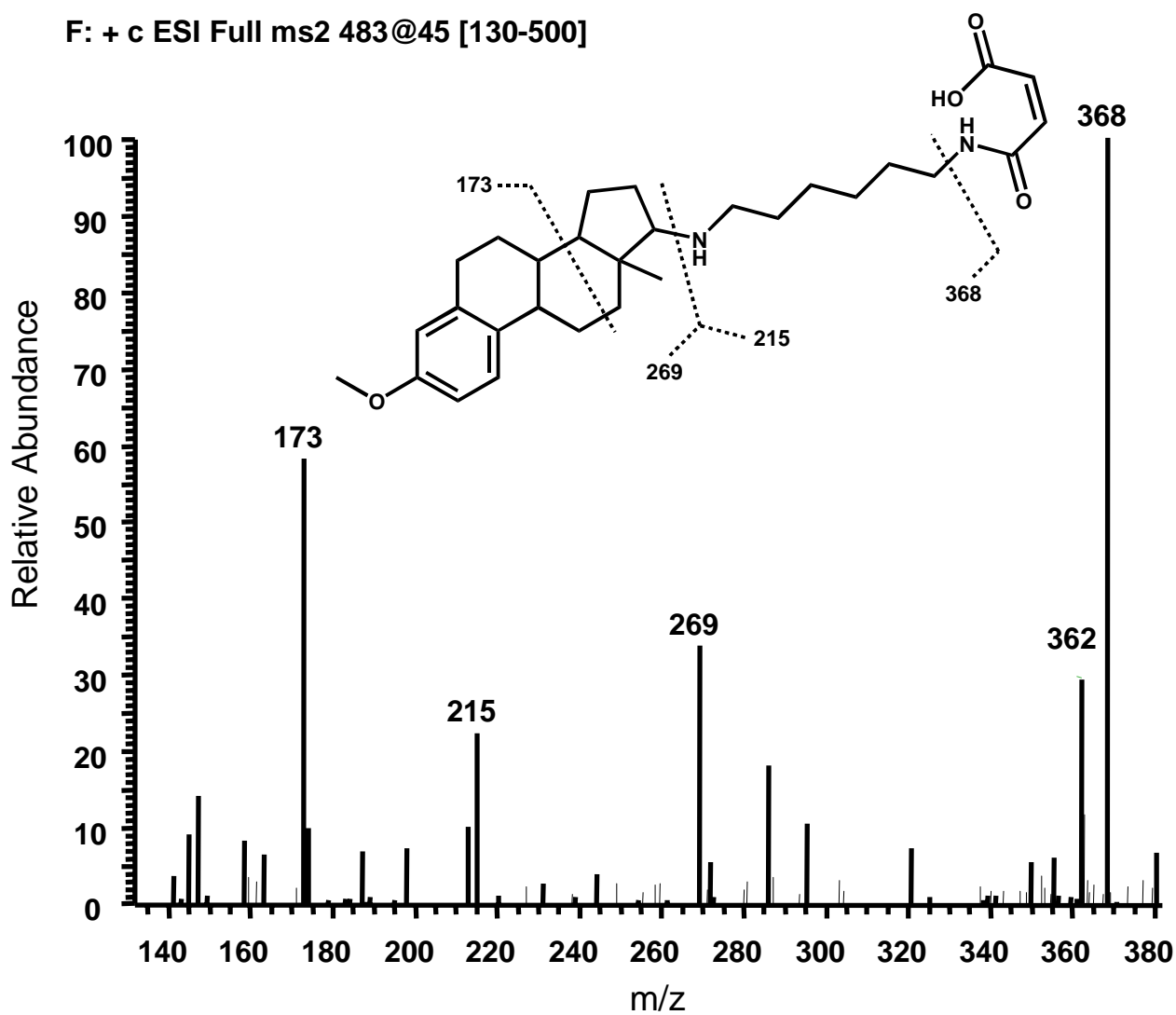
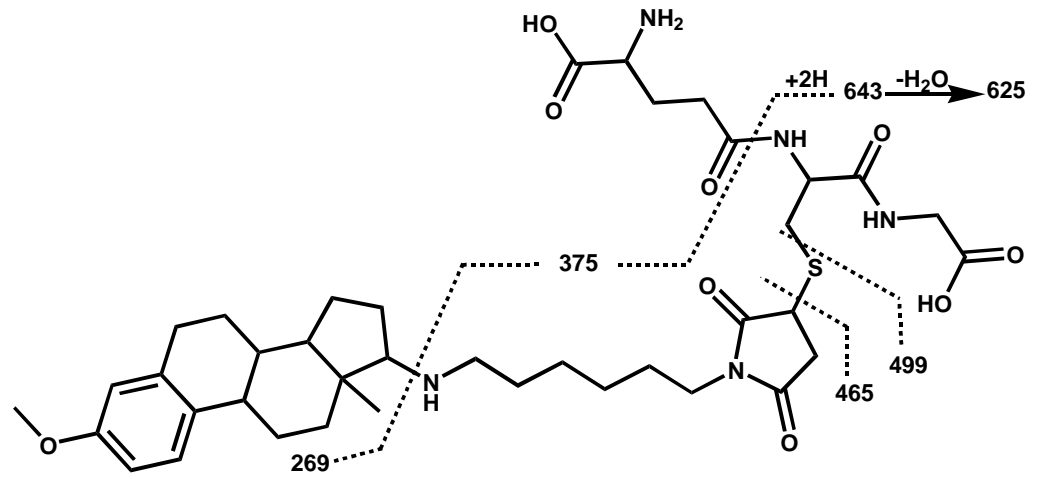


FIGURE 3B

C



F: + c ESI Full ms3 772@20 643@32 [175-800]

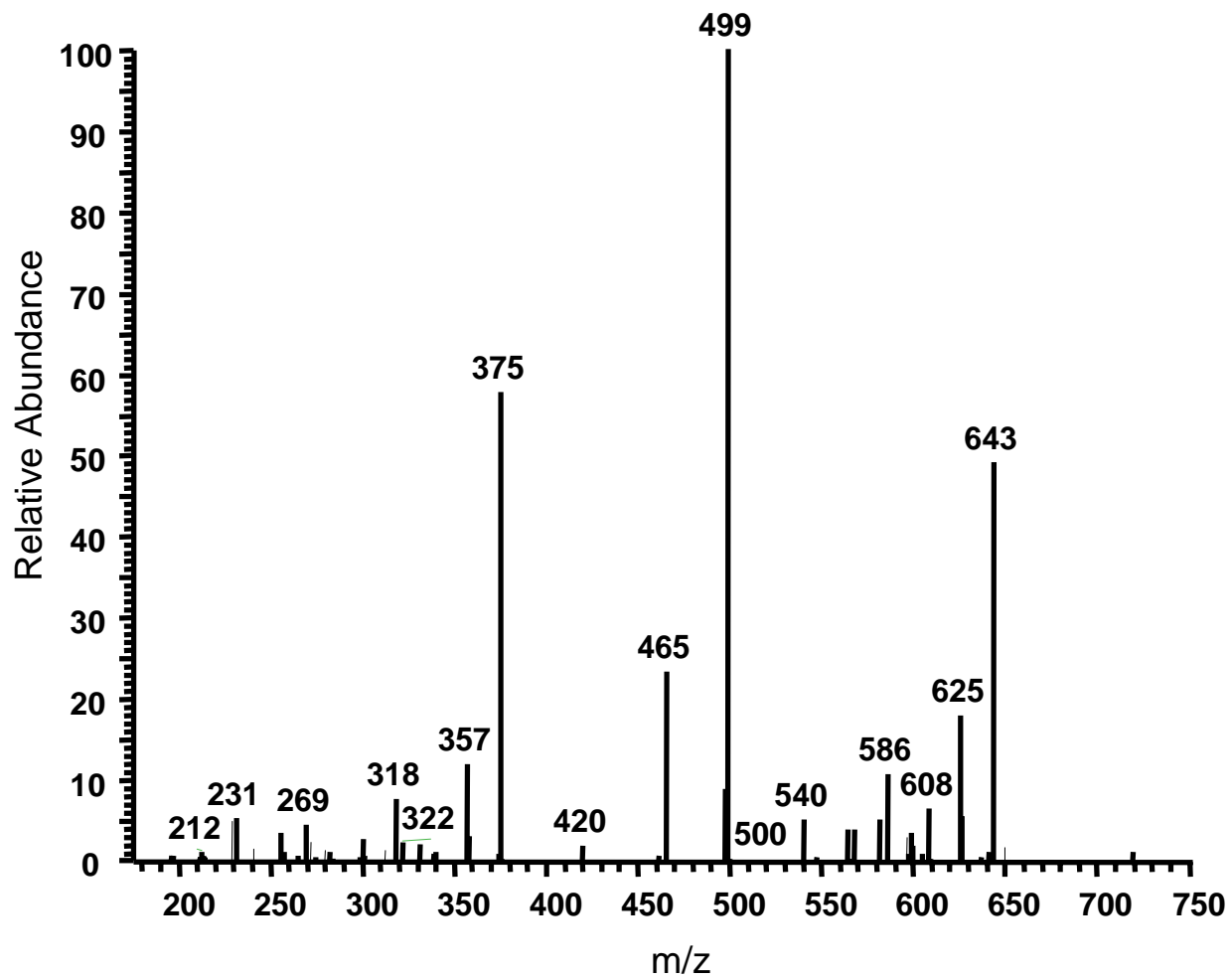


FIGURE 3C

D

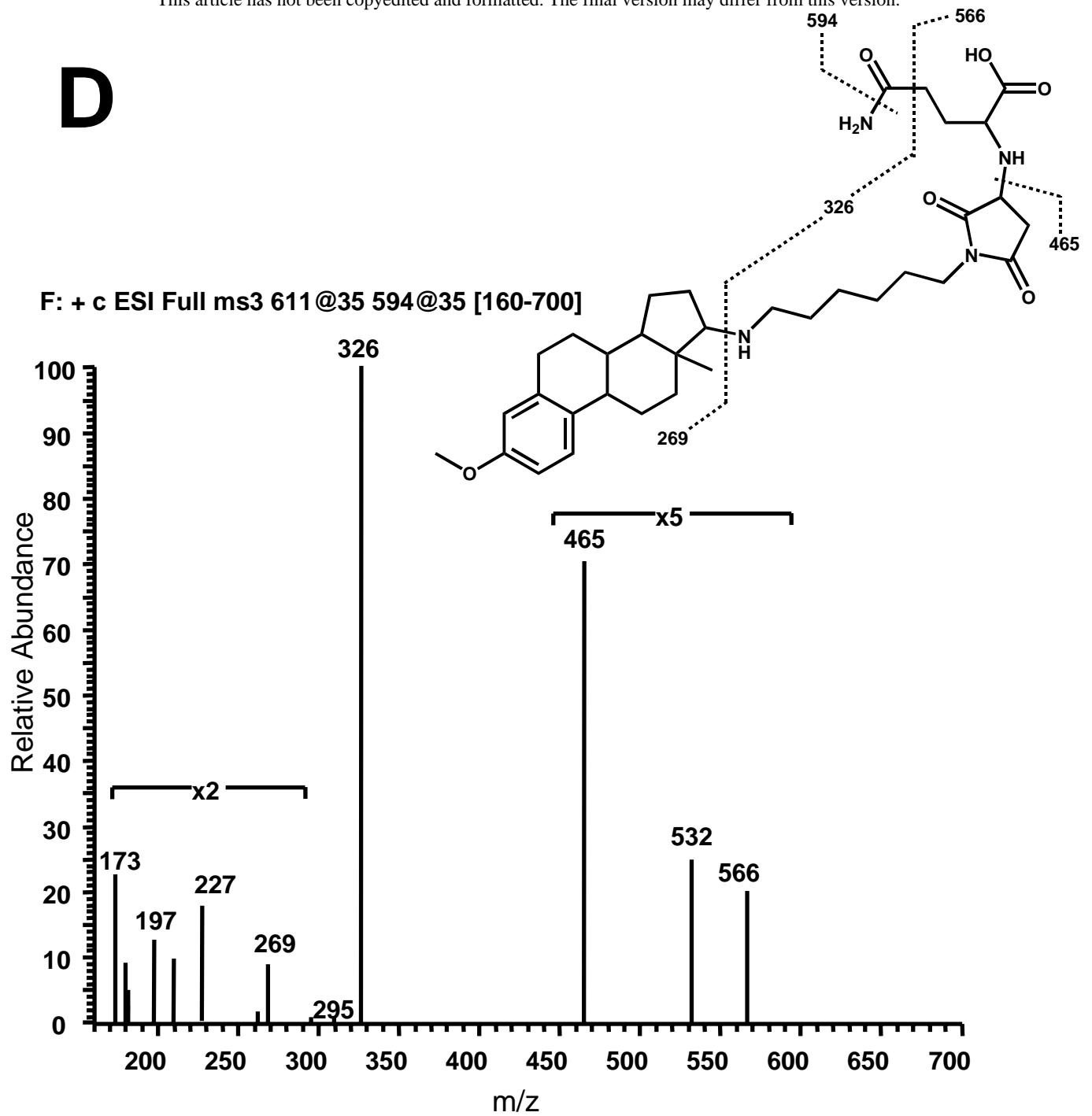
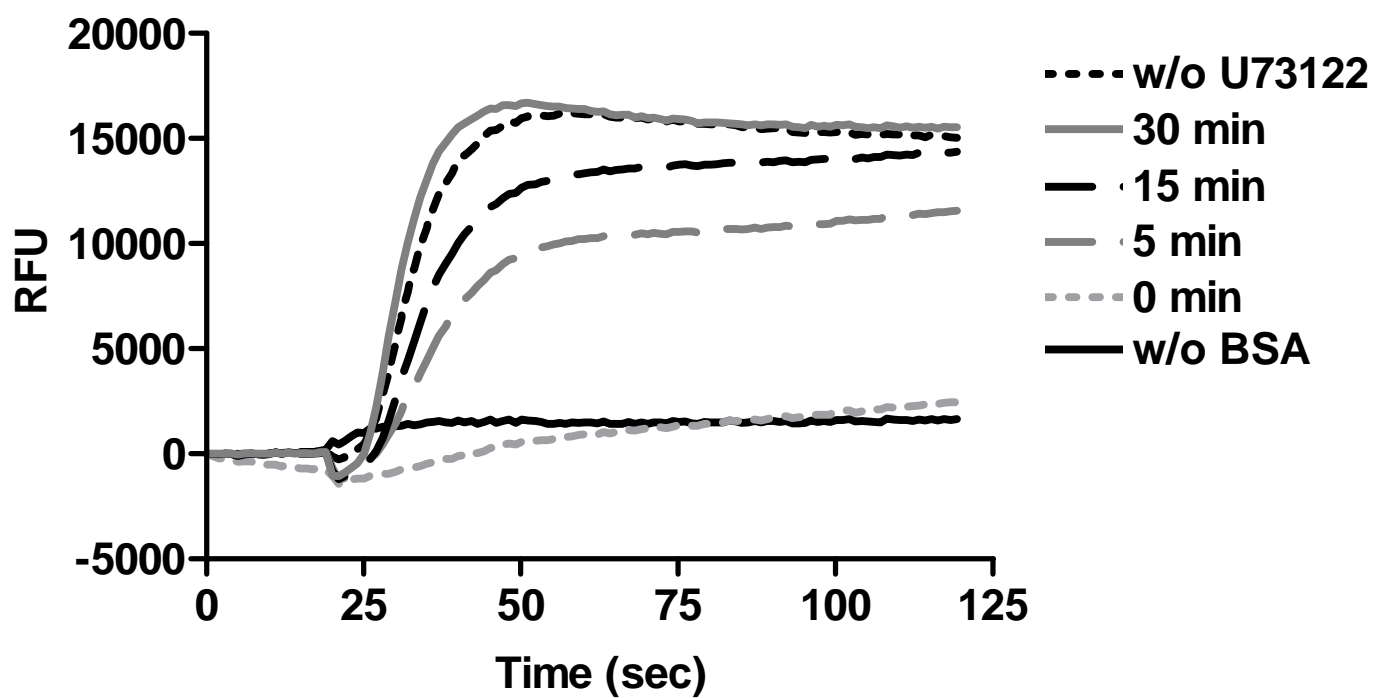


FIGURE 3D



**A**



**B**

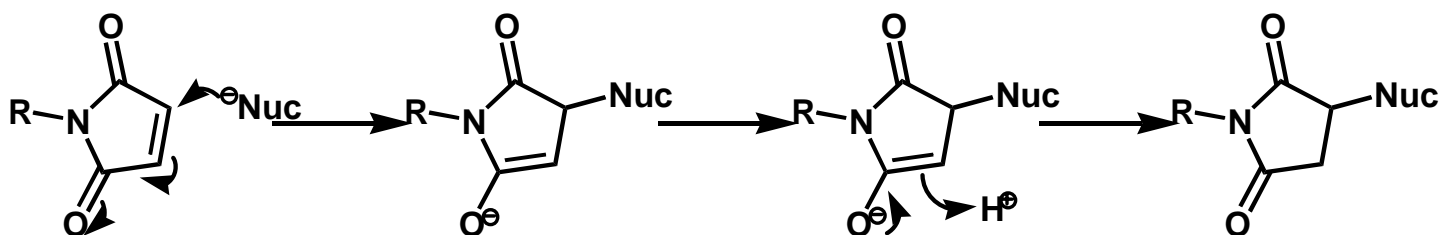


FIGURE 4