

Synthesis and Fragmentation Behavior Study of *n*-alkyl/benzyl Isatin Derivatives Present in Small/Complex Molecules: Precursor for the Preparation of Biological Active Heterocycles

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Abstract: *N*-Alkyl/benzyl substituted isatin derivatives are intermediates and synthetic precursors for the preparation of biological active heterocycles. *N*-alkyl/benzyl isatins have showed various biological activities, such as cytotoxicity, antiviral, caspase inhibition, cannabinoid receptor 2 agonists for the treatment of neuropathic pain, etc. In this study, *N*-alkyl/benzyl isatin derivatives were synthesized from isatin and alkyl/benzyl halides in presence of K_2CO_3 in DMF and excellent to quantitative yields (~95%) were obtained. Isatins and benzyl-isatins were condensed with fluorescein hydrazide to form fluorescein hydrazone. All the compounds were subjected to their fragmentation behavior study using LC/MSⁿ. *N*-Alkyl substituted isatin derivatives fragmented at nitrogen-carbon (N-C) bond, hence gave daughter ion as $[RN+H]^+$. Whereas, *N*-benzyl substituted isatin derivatives fragmented at carbon-carbon (C-C) bond of alkyl chain which linked with nitrogen molecules, therefore gave *N*-methyl fragments $[RNCH_2]^+$. This study demonstrated that, isatin moiety present in a small/large molecule or in a matrix of reaction mixture with/without *N*-alkyl/benzyl substituents can be identified by mass spectroscopic fragmentation behavior study.

Key words: Isatin, *N*-Alkyl/aryl isatin, Fragmentation behavior, Heterocycles

Introduction

Isatin (**1**, 1*H*-indole-2,3-dione) is an indole derivative which was first obtained by oxidation of indigo dye,^{1,2} later on, it was synthesized by condensation of chloral hydrate, aniline and hydroxylamine in sulphuric acid which is so called Sandmeyer isonitrosoacetanilideisatin synthesis.^{3,4} Isatin is occurring in *Isatis tinctoria*,⁵ *Calanthe discolor*,⁶ in *Couroupita guianensis*⁷ and many other sources.⁸⁻¹⁰ It has been used to test the presence of thiophene in crude benzene.¹¹ Large number of biological active molecules which consist of isatin moiety showed various biological activities, such as anticonvulsant, antiviral, free radical scavengers, cytotoxic, antitumor, antineoplastic, antileishmanial, enhancer of ATRA-induced differentiation, ADAMTS-5 (Aggrecanase-2) inhibitors and inhibitors of NADPH oxidase (Chart 1).

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Thus, synthesis and study of isatin derivatives have been increasingly promoted in the scientific area. *N*-Alkyl/benzyl isatins can be prepared by using NaH / K_2CO_3 / CaH_2 / F_3CCOOH / $EtN(i-Pr)_2$ / Et_3N / C_5H_5N / $POCl_3$ / $LiAlH_4$ / KOH / etc. as reagents.

Due to the intriguing structures of isatin and its derivatives as well as their presence in the various forms in natural / synthetic biological active molecules, we focused on how to figure out the presence of isatin, *N*-alkyl/benzyl isatins moiety in natural / synthetic molecules before isolating from natural sources and/or purifying the reaction mixtures. In order to achieve this goal, we need to study the

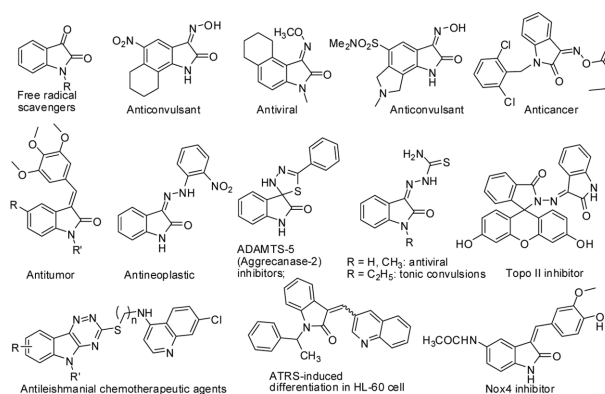


Chart 1. Biological active compounds containing isatin skeleton.

properties of the compounds of our interests. Since our group has been heavily involving in the field of mass spectroscopic research,¹²⁻¹⁵ we have designed and synthesized a series of *N*-alkyl/benzyl isatin derivatives and studied their fragmentation behavior using LC/MSⁿ. These studies turned out to give us a solution to identify the presence of isatin moiety in natural / synthetic molecule before isolating the bioactive compounds from natural sources or before purifying the reaction mixtures. In addition, we can apply this method to indentify complex compounds containing the bioactive skeletons in natural source.

Experimental

General

Chemicals and solvents were commercial reagent grade and were used without further purifications. Melting points were determined on a Barnstead electrothermal digital melting point apparatus model 9100 and are uncorrected. NMR spectra were obtained using a Bruker-500 spectrometer and Agilent technologies 400MR spectrometer. Electrospray ionization (ESI) mass spectrometry (MS) experiments were performed using an Agilent high performance liquid chromatography (HPLC) 1200 connected to an Agilent 6320 ion trap mass spectrometer fitted with an electrospray ionization (ESI) ion source (Agilent Technologies, Palo Alto, CA, USA). Agilent 6300 series ion trap LC/MS system software, Version 6.2, was used for data acquisition and analysis. Direct injection was performed using a connector. The mobile phase was composed of two solvents, methanol and water with 1:1 ratio. Flow rate was 0.3 mL/min, run time was 3 min. The ESI ion source capillary temperature was set at 325°C; the dry gas flow rate was set to 10.0 L/min; nebulizer pressure was set to 40.00 psi; maximum accumulation time was 300,000 μ s; spectra were taken in positive/negative mode and scan began at 50 *m/z* and ended at 500 *m/z*; the injection volume was 2 μ L.

General procedure for the synthesis of *N*-substituted isatin (2a-i).

A flask equipped with a magnetic stirring bar was charged with DMF (100 mL) and potassium carbonate (13 mmol). The mixture was stirred at room temperature for 5 min., isatin (1,10 mmol) was then added and the stirring was continued for 45 min. Alkyl/benzyl halide (11 mmol, chloride or bromide) was added to the reaction mixture and the stirring was continued at 80°C for 12 h. The mixture was then diluted with water (200 mL), extracted with ethyl acetate and dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was purified by flash column chromatography on silica gel using ethyl acetate and hexane (1:9) solvent system. Analytical pure *N*-substituted isatin was obtained in quantitative yields. 1-Methyl-1*H*-

indole-2,3-dione (**2a**). Orange solid (90%). Mp 133-134°C [lit.¹⁶ mp. 132-134°C; lit.¹⁷ mp. 133°C]. MS (ESI): Calculated 161, found 162 [M+H]⁺; 1-Ethyl-1*H*-indole-2,3-dione (**2b**). Yellowish-orange solid (96%). Mp. 161-162°C [lit.¹⁸ mp. 162-163°C]. MS (ESI): Calculated 175, found 176 [M+H]⁺; 198 [M+Na]⁺; 215 [M+K]⁺; 1-Propyl-1*H*-Indole-2,3-dione (**2c**). Orange solid (92%). Mp. 61-62°C [lit.¹⁶ mp. 62 -62°C]. MS (ESI): Calculated 189, found 212 [M+Na]⁺; 1-Butyl-1*H*-Indole-2,3-dione (**2d**). Red thick oil (85%) [lit.¹⁹ mp. 36°C]. MS (ESI): Calculated 203, found 204 [M+H]⁺; 226 [M+Na]⁺; 1-Hexyl-1*H*-Indole-2,3-dione (**2e**). Yellow low melting solid (85%) [lit.¹⁶ mp. 39 - 40°C]. MS (ESI): Calculated 231, Found 254 [M+Na]⁺; 1-(*iso*-Butyl)-1*H*-Indole-2,3-dione (**2f**).¹⁹ Red solid (89%). Mp. 90-91.C [lit.²⁰ mp. 88-89°C]. MS (ESI): Calculated 203, found 204 [M+H]⁺; 1-(*sec*-Butyl)-1*H*-Indole-2,3-dione (**2g**).²¹ Red low melting solid (87%). MS (ESI): Calculated 203, found 204 [M+H]⁺; 1-Benzyl-1*H*-Indole-2,3-dione (**2h**). Orange solid (95%).^{22,23} Mp 130-131°C [lit.¹⁷ mp. 122-123°C; lit.²⁴ mp. 129-131°C]. MS (ESI): Calculated 237, Found 238 [M+H]⁺; 260 [M+Na]⁺; 1-(3-Methoxybenzyl)-1*H*-Indole-2,3-dione (**2i**).^{9,10} Orange solid (82%). Mp 109-110°C. MS (ESI): Calculated 267, Found 268 [M+H]⁺; 290 [M+Na]⁺.

General procedure for the synthesis of isatin derivatives (3a-b)

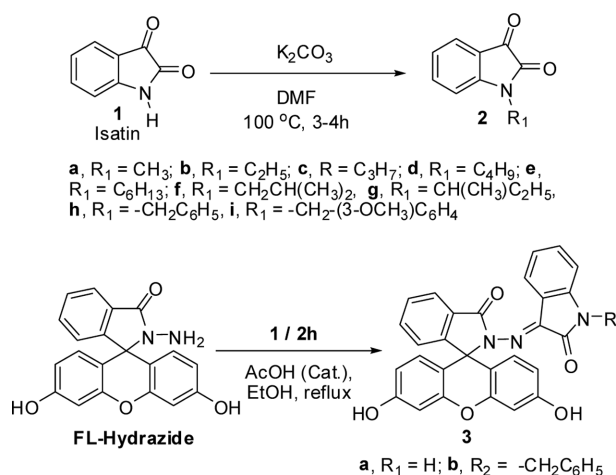
A mixture of fluorescein hydrazide (**2**, 0.3 mmol) and substituted isatins (0.3 mmol) were reacted according to the reported method.²⁵ (E)-3',6'-dihydroxy-2-(2-oxoindolin-3-ylideneamino)spiro[isindoline-1,9'-xanthen]-3-one (**3a**). Orange solid; (98%); mp. 261°C [lit.²⁵ mp. 261°C]. MS (ESI): Calculated 475, Found 476 [M+H]⁺; 498 [M+Na]⁺; (E)-2-((1-benzyl-2-oxoindolin-3-ylidene)amino)-3',6'-dihydroxyspiro[isindoline-1,9'-xanthen]-3-one (**3b**). Orange solid; (99%); mp. 269°C. ¹H NMR (DMSO-*d*₆, 400 MHz): δ , 9.93 (s, 2H), 7.98 (d, *J* = 6.8 Hz, 1H), 7.68 (td, *J* = 7.8, 1.2 Hz, 1H), 7.63 (td, *J* = 7.8, 1.2 Hz, 1H), 7.36 (td, *J* = 7.8, 1.2 Hz, 1H), 7.34-7.28 (m, 4H), 7.28-7.24 (m, 1H), 7.21 (d, *J* = 7.8 Hz, 1H), 7.17 (d, *J* = 7.8 Hz, 1H), 7.04 (t, *J* = 7.8 Hz, 1H), 6.98 (d, *J* = 7.8 Hz, 1H), 6.60 (d, *J* = 2.4 Hz, 2H), 6.58 (d, *J* = 8.8 Hz, 2H), 6.44 (dd, *J* = 8.4Hz, 2H), 4.85 (s, 2H) ppm. ¹³C NMR (DMSO-*d*₆, 100 MHz): δ 163.01, 160.50, 159.05, 152.60, 151.80, 151.08, 136.40, 134.90, 133.85, 129.80, 129.20, 128.91, 128.48, 128.42, 128.06, 127.85, 124.54, 124.47, 123.58, 117.13, 112.90, 110.28, 110.21, 102.84, 67.65 and 43.28 ppm. MS (ESI): Calculated 565, Found 566 [M+H]⁺; 588 [M+Na]⁺.

Results and Discussion

N-Substituted alkyl/benzyl isatin derivatives (**2a-i**) were synthesized from commercially available isatin (**1**) in presence of potassium carbonate (K₂CO₃) in DMF at 80°C. Condensation of isatin (**1**) / *N*-benzylisatin (**2h**) with FL-

Hydrazone using literature procedure¹⁶ resulted in compounds **3a-b**. (Scheme 1).

Isatin (**1**) was first introduced into the ESI ion source of Ion trap mass spectrometer by direct injection using methanol and water solvent system. Molecular ion peak were obtained m/z at 148 in positive mode as $[M+H]^+$ (100%) (Figure 1A) and m/z at 146 in negative mode as $[M-H]^-$ (100%) (Figure 1B). Fragmentation (MS^2) of **1** gave the product ion at m/z 120 with the loss of carbon monoxide (CO) (Figure 1C).



Scheme 1. Synthesis of *N*-substituted isatin derivatives (**2a-i**, **3a-b**)

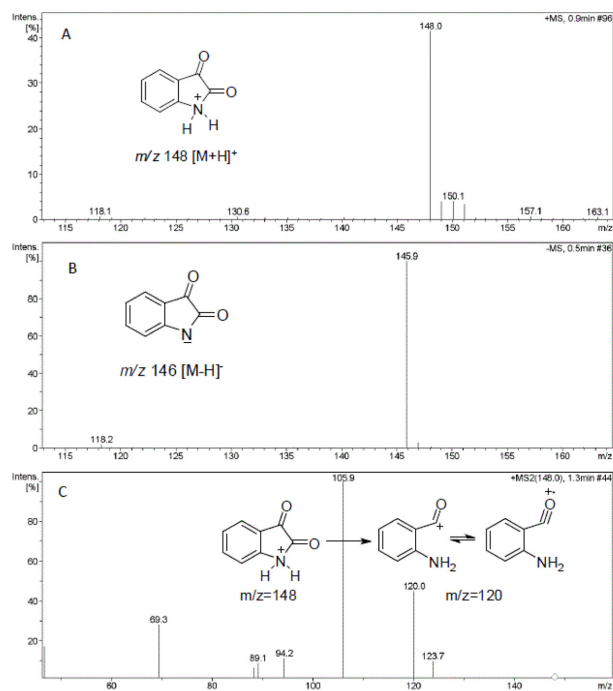


Figure 1. Mass spectra of Isatin (MS^2 scan and MS^2 fragmentation): A) in positive mode $m/z = 148$; B) in negative mode $m/z = 146$; C) fragmentation (MS^2) gave $m/z = 120$.

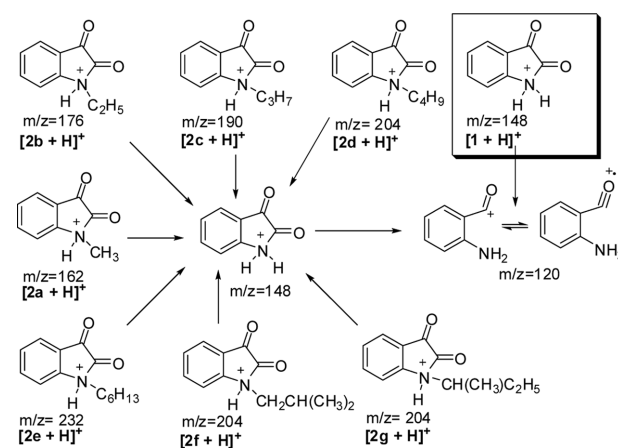
For the fragmentation behavior study of *N*-alkyl/benzyl isatin derivatives (**2a-i**), first, multistage mass spectral data (MS^2 and MS^3) were taken for *N*-substituted alkyl isatin derivatives (**2a-g**). It elucidates that, MS^2 of compounds **2a-g** gave m/z at 148 (100%) with the loss of alkyl group attached with nitrogen atom of isatin skeleton.

Further fragmentation (MS^3) of m/z 148 ion gave the product ion at m/z 120. Details of fragmentation behavior study are depicted in scheme 2.

Scheme 2. Proposed MS^n (MS^2 and MS^3) mechanism pattern of *N*-alkyl substituted isatin (**2a-g**).

It should be noted that, all the compounds **2a-g** gave fragments at m/z 148 with the loss of substituted alkyl group and the alkyl chain did not affect the fragmentation behavior in compounds **2a-g** (Figure 2).

MS^2 fragment at m/z 148 obtained from parent compounds (**2a-g**) were extensively studied for their MS^3 fragmentation behavior. All the fragments had m/z at 120 (100%) with the loss of one molecule of carbon monoxide



Scheme 2. proposed MS^n (MS^2 and MS^3) mechanism pattern of *N*-alkyl substituted isatin (**2a-g**).

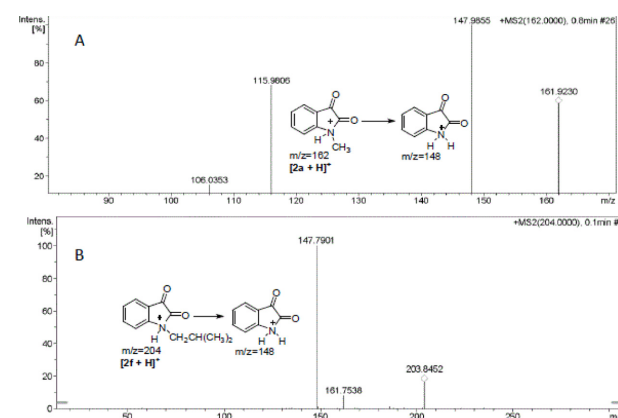


Figure 2. MS^2 Fragmentation behavior of substituted *N*-alkyl isatin (**2a** and **2f**): A) *N*-methyl isatin (**2a**); B) *N*-iso-butyl isatin (**2f**).

(CO) while some of them gave m/z at 92 (0-60%) with the loss of two molecule of carbon monoxide (CO) along with m/z at 120 (100%). Figure 3 shows a short chain substituent *N*-alkyl isatin, namely *N*-ethyl isatin (**2b**, Figure 3A), long chain substituent *N*-alkyl isatin, *N*-hexyl isatin (**2e**, Figure 3B) and branched chain substituent *N*-alkyl isatin, *N*-*iso*-butyl isatin (**2f**, Figure 3C).

Multistage mass spectral data (MS^2 and MS^3) were also taken for *N*-benzyl isatin derivatives (**2h-i**) and different fragmentation behavior was observed than the *N*-alkyl isatin derivatives (**2a-g**) (Scheme 3).

MS^2 fragmentation of compound **2h** gave three fragments, m/z 160: with the loss of benzyl group attached with nitrogen atom of isatin skeleton, m/z 132: might be attributed to the loss of carbon monoxide generated from fragment ion m/z 160, and m/z 91: most likely generated from phenyl ring attached with nitrogen atom by methylene

(-CH₂) chain. To our notice, the fragment at m/z 91 is a common tropylium ion or phenylmethylium ion generated from parent compound **2h** (Figure 4A).

Similarly MS^2 fragmentation of compound **2i** having 3-methoxy group attached with phenyl ring gave three fragments, m/z 160: with the loss of benzyl group attached with nitrogen atom of isatin skeleton, m/z 132: might be attributed to the loss of carbon monoxide generated from fragment ion m/z 160, and m/z 121: most likely generated from 3-methoxy phenyl ring attached with nitrogen atom by methylene (-CH₂) chain. The fragment at m/z 121 is also a common tropylium ion or phenylmethylium ion generated from parent compound **2i** (Figure 4B).

MS^2 fragment at m/z 160 obtained from parent compounds **2h-i** were extensively studied for their MS^3 fragmentation behavior in a similar fashion as for **2a-g**. Both fragments at m/z 160 gave m/z 132 (100%) with the

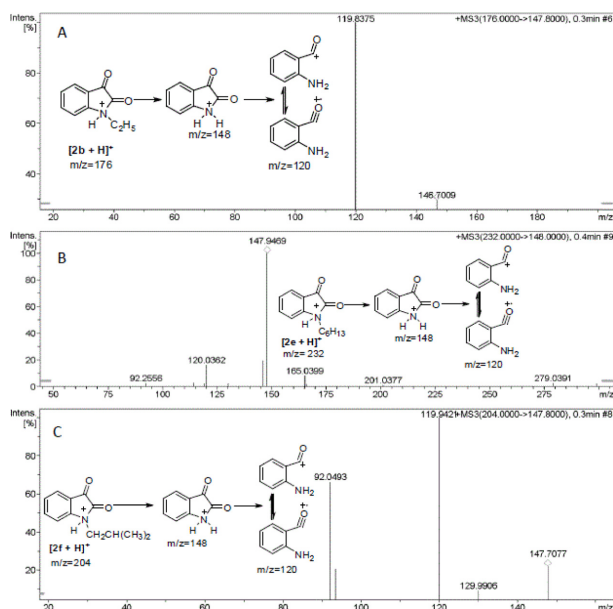
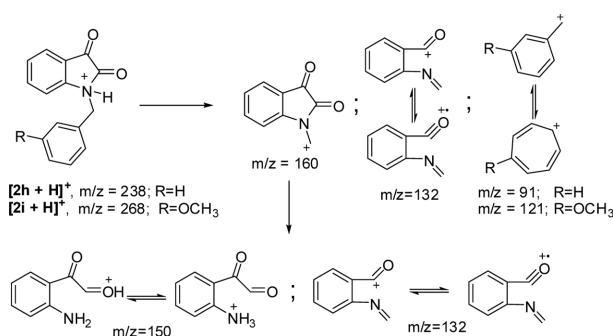


Figure 3. MS^3 Fragmentation behavior of substituted *N*-alkyl isatin (**2b**, **2e** and **2f**): A) *N*-ethyl isatin (**2b**); B) *N*-*n*-hexyl isatin (**2e**); C) *N*-*iso*-butyl isatin (**2f**).



Scheme 3. Proposed MS^n (MS^2 and MS^3) mechanism behavior of *N*-benzyl substituted isatin (**2h-i**)

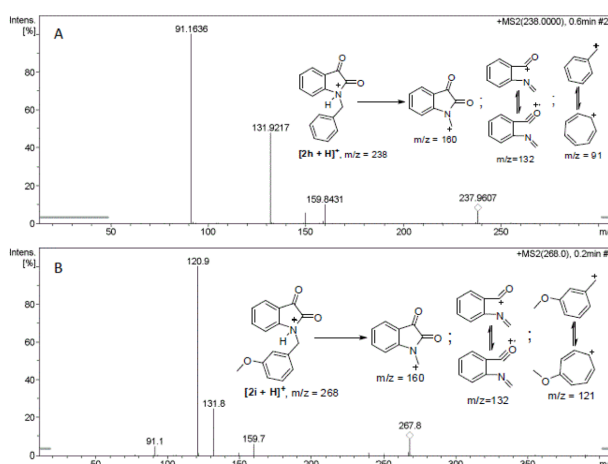


Figure 4. MS^2 Fragmentation behavior of substituted *N*-benzyl isatin (**2h-i**): A) *N*-phenyl isatin (**2h**); B) *N*-(3-methoxy)phenyl isatin (**2i**).

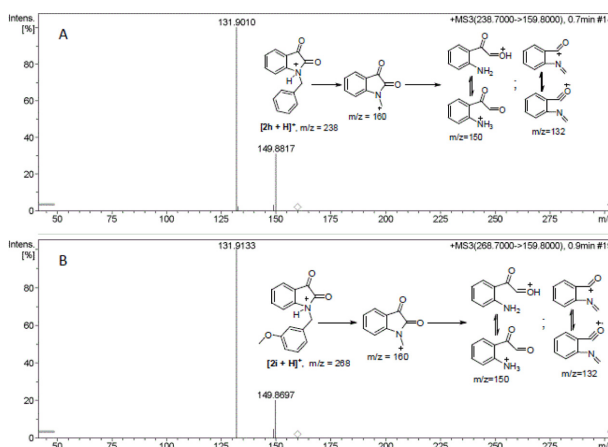
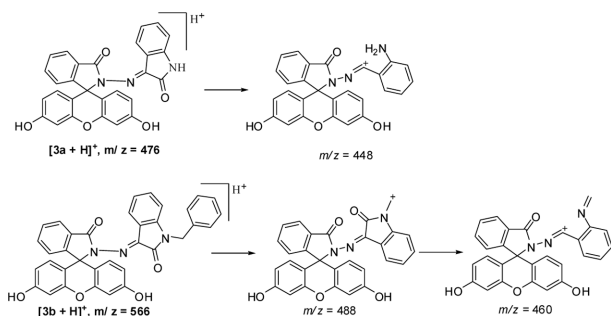


Figure 5. MS^3 Fragmentation behavior of substituted *N*-benzyl isatin (**2h-i**): A) *N*-phenyl isatin (**2h**); B) *N*-(3-methoxy)phenyl isatin (**2i**).

Synthesis and Fragmentation Behavior Study of *n*-alkyl/benzyl Isatin Derivatives Present in Small/Complex Molecules:



Scheme 4. Proposed MSⁿ mechanism behavior of fluorescein hydrazone isatin/benzyl isatin derivatives (**3a-b**)

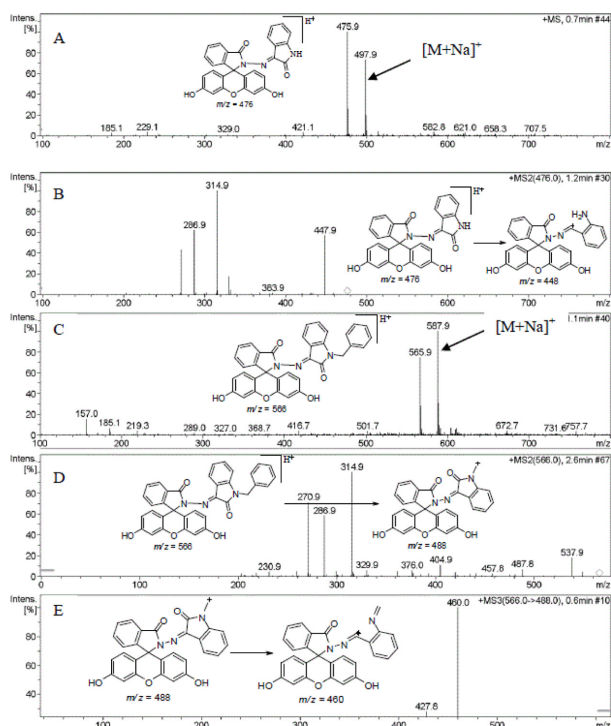


Figure 6. Mass spectra of compounds **3a-b**: A) m/z 476 [M+H]⁺ and m/z 498 [M+Na]⁺ of **3a**; B) MS² of m/z 476 gave fragment at m/z 448; C) m/z 566 [M+H]⁺ and m/z 588 [M+Na]⁺ of **3b**; D) MS² of m/z 566 gave fragment at m/z 488; E) MS³ of m/z 488 gave fragment at m/z 460.

loss of one molecule of carbon monoxide (CO) along with m/z 148 (20-30%) with the loss of methane. Figure 5 shows *N*-benzyl isatin (**2h**, Figure 5A) and *N*-(3-methoxy)benzyl isatin (**2i**, Figure 5B), respectively.

Since one of the main objectives of our study is to identify if the isatin moiety presents in a complex molecules or in a fraction of natural extracts by using this fragmentation behavior method, we therefore applied this method to identify isatin moiety present in a complex fluorescein hydrazone isatin/benzylisatin derivatives, namely (E)-3',6'-dihydroxy-2-((2-oxoindolin-3-ylidene)amino)spiro[isindoline-1,9'-xanthen]-3-onen (**3a**)¹⁶ and (E)-2-((1-be-

nzyl-2-oxoindolin-3-ylidene)amino)-3',6'-dihydroxyspiro[isindoline-1,9'-xanthen]-3-one (**3b**), respectively.

As shown in Scheme 4, compounds **3a-b** gave same pattern as we expected. From figure 6A, we can see that, compound **3a** gave m/z 476 [M+H]⁺ and sodium adduct at m/z 498 [M+Na]⁺. MS² fragmentation of m/z 476 gave fragment at m/z 448 with the loss of one molecule of carbon monoxide from the parent (Figure 6B). As for benzyl isatin moiety containing compound **3b**, the exactly same fragmentation pattern as for small molecules like **2h-i** were observed. It showed m/z at 566, MS² fragmentation of m/z 566 gave m/z at 488 with the loss of benzyl group attached with nitrogen atom of isatin skeleton, and in MS³ fragmentation of daughter fragment m/z 488 gave m/z at 460 with the loss of carbon monoxide. Details of mass spectra are shown in Figure 6.

From the Figure 6, conclusion can be drawn that, isatin moiety (with / without substituent) presents in small / large or complex mixtures its gives similar fragmentation patterns.

Conclusions

A series *N*-alkyl/benzyl isatin derivatives (**2a-i**) and two fluorescein Isatin hydrazone (**3a-b**) were synthesized and subjected to their mass spectroscopic fragmentation behavior study using Ion Trap LC/MS. Fragmentation of *N*-alkyl substituted isatin derivatives gave daughter ion as [Rⁿ+H]⁺ hence the breakage occurred nitrogen-carbon (N-C) bond, whereas fragmentation of *N*-benzyl substituted isatin derivatives gave *N*-methyl fragments [RNCH₂]⁺, hence the breakage occurred in carbon-carbon (C-C) bond of alkyl chain. Same fragmentation behavior were observed for the complex molecule fluorescein hydrazone (**3a-b**). Based on this fragmentation behavior pattern, whether isatin moiety present or not in a small/large molecule or in a matrix of reaction mixture with or without substituents can be easily identified. Further studies about this method for identification of basic skeleton in natural mixture are in progress.

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