FACILE ROUTE TO AMINO PHTHALIMIDES AND ISOTHIOCYANATE ANALOGUES; NOVEL REAGENTS TO PREPARE FLUORESCENT PROTEIN CONJUGATES.

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Abstract: A simple synthesis of 3 or 4-aminophthalimides as well as the corresponding isothiocyanates starting from phthalic anhydride is described; these molecules are suggested as new reagents to obtain fluorescent protein conjugates. Additionally a mass spectrometric study for the target compounds was performed.

Introduction. Fluorescein isothiocyanate (**FI**) is a compound that offers optimum properties for fluorescence immunoassay, since in addition to its fluorescence (1), it easily forms complexes with immune substances such as antibodies and antigens among others, being able to be detected in this way (2,3). However the synthesis of **FI** is a multiple step process which raises its price and consequently limits its use (4).

This work deals with the synthesis of four fluorescent molecules; the 3 and 4 aminophthalimides (**3a-b**) and their correspondent isothiocyanate derivatives (**4a-b**). The target compounds were produced following, by analogy those synthesis published for luminol (5,6) and **FI**, which used phthalic anhydride as starting material (scheme 1). In addition to their fluorescence properties these molecules have an amino or isothiocyanate group which probably leads to conjugation with immune substances (7), thus immunological tests have precedents. It is worth to mention that to our knowledge the molecules **3-4** do not have precedents as immunological reagents. Additionally a mass spectrometric study for the phthalimide moieties was performed; thus, electron impact mass spectrometry (EIMS), collision induced dissociation at constant B/E (CID-Linked-scans) and high resolution mass spectrometry (HRMS) were employed.

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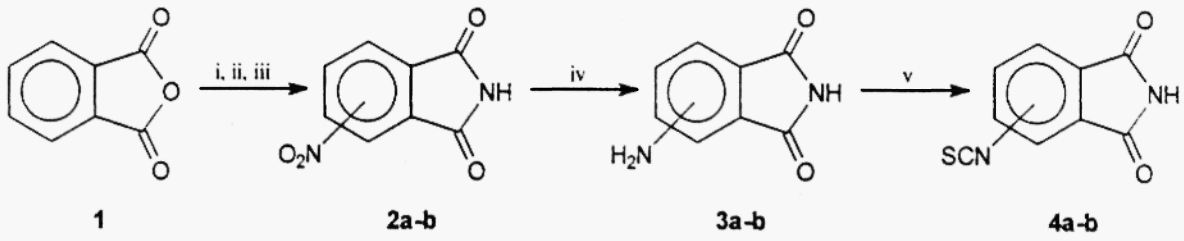


Scheme 1. *Reagents and conditions:* i, HNO_3; ii, Ac_2O/Δ; iii, NH_4OH/Δ; iv, H_2, Pd/C, MeOH, RT., 1 atm.; v, $CSCl_2$/AcOH/10% HCl,

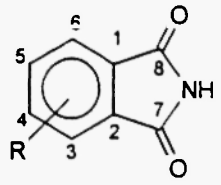
Results and Discussion

Synthesis

In search of new possible options for fluorescent protein conjugates (**3a-b** and **4a-b**) we have explored the synthetic route showed in scheme 1. The starting point for the synthesis of the target compounds was the preparation of the nitro phthalimides **2a** and **2b** from phthalic anhydride using previously reported procedures (8). The corresponding nitro intermediates were then reduced to the respective amino derivatives, **3a** 75.8 % and **3b** 63.1 %, by means of a catalytic hydrogenation. Then, when **3a-b** were treated with thiophosgene under biphasic conditions the corresponding isothiocyanates **4a-b** were achieved; probably by means of the aminothiocarbonyl chlorides and their corresponding dehydrohalogenation, no thioureas were detected (9).

Mass spectra

For the structural attribution of **3a-b** and **4a-b** the corresponding NMR assignments, see experimental and figure 1, were in agreement with those obtained by means of a mass spectrometric study.



R = NH_2, N=C=S

Figure 1

Thus, a general fragmentation pattern for the molecules under study was proposed (scheme 2), which is complementary to the observed key ions in the EI mass spectra of the target compounds compiled in table 1. The ion structures shown in the fragmentation scheme are theoretical in principle, and have been presented here as a framework for the fragmentation pathways that we are suggesting. Two symbols in the scheme mark arrows of some of the pathways investigated by unimolecular linked scans at constant B/E and by high resolution data.

This pattern was proposed based on the high resolution data for the ion M^+ of the four compounds, as well as for the main fragments originated from the molecular ion of **4**a and its corresponding daughter ions (table 2) acquired by CID-linked scans (10), a method of ion characterization employed to determinate the origin of a peak (daughter) from a precursor fragment.

As can be seen (table 1) the relative abundance of the molecular ions in the title compounds corresponds to the base peaks reflecting their stability. Moreover since the measurement of the mass of an ion with sufficient accuracy provides an unequivocal identification of its elemental composition the corresponding data (table 3) were determined employing HRMS. Consequently it could be stated that the species **a**, lost the neutral fragment CHON of mass 43 to achieve the ion **b** which also arrived from **a'** explained by the rearrangement (11) of a. The successive loss of CO_2 from **a**, provided the fragment **c** (10-16 % of ra), and finally the specie **d** was promoted directly from **b** and **c** by the loss of CO and CNH respectively which in turn achieved the ions m/z 63 (**f**) and m/z 75 (**g**).

Table 1. Approximate relative abundance (% base peak) of main ions in the 70 eV mass spectra of phthalimides **3a-b** and **4a-b**.

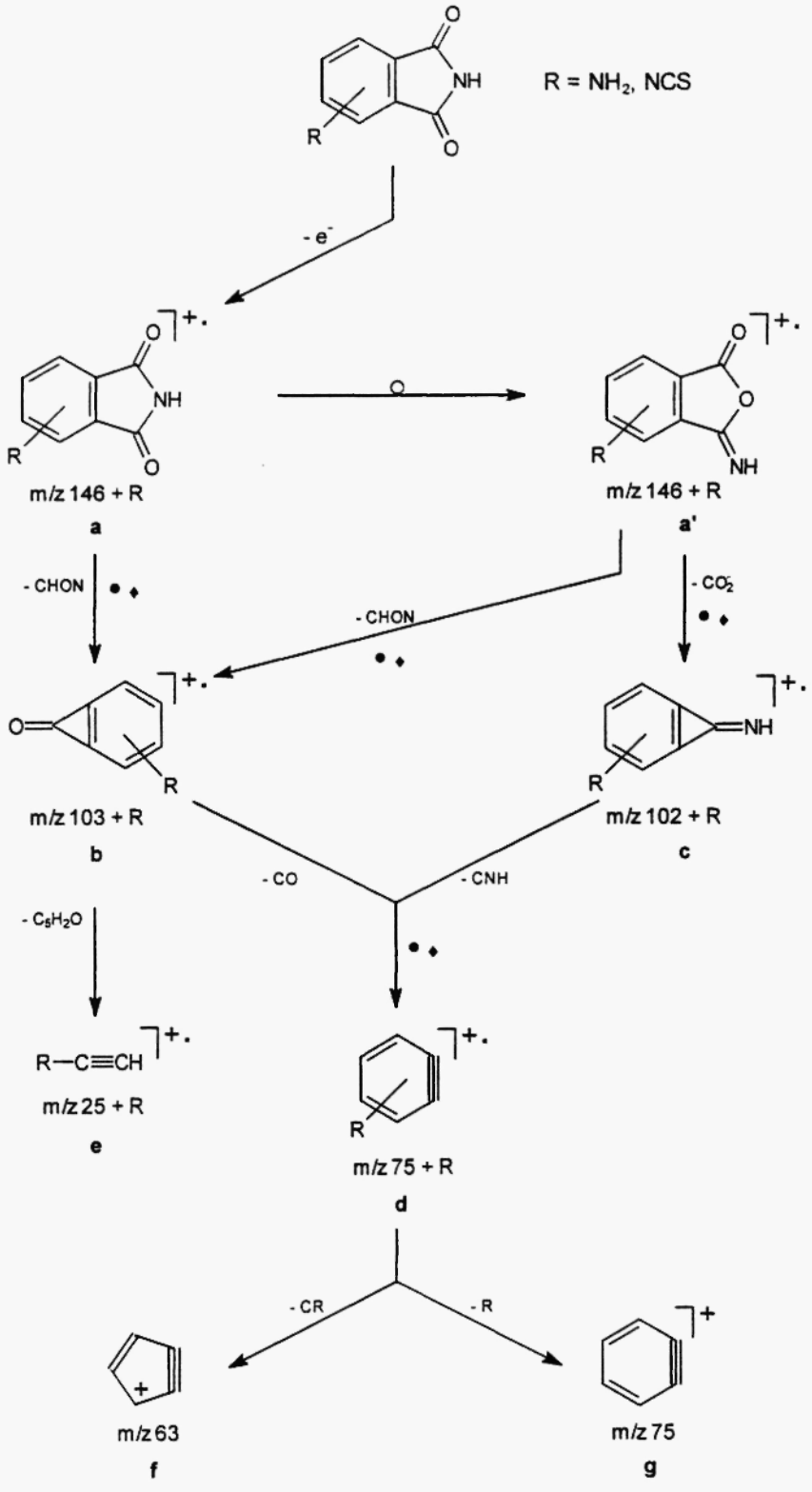
General ion	Assignment	3a	3b	4a	4b
a	$M^{+\cdot}$	162 (100)	162 (100)	204 (100)	204 (100)
b	$[M\text{-}CHON]^{+\cdot}$	119 (9)	119 (29)	161 (30)	161 (22)
c	$[M\text{-}CO_2]^{+\cdot}$	118 (12)	118 (12)	160 (16)	160 (10)
d	$[M\text{-}CHON\text{-}CO]^{+\cdot}$	91 (30)	91 (37)	133 (31)	133 (22)
e	$[M\text{-}CHON\text{-}C_5H_2O]^{+\cdot}$	41 (2)	41 (10)	83 (2)	83 (5)
f	$[M\text{-}CHON\text{-}CO\text{-}CR]^{+}$	63 (5)	63 (9)	63 (2)	63 (2)
g	$[M\text{-}CHON\text{-}CO\text{-}R]^{+}$	75 (1)	75 (1)	75 (13)	75 (5)

Table 2. Daughter ions generated by CID (Linked scans), and high resolution data for the main fragments of molecule **4a**.

Parent ions	204.1157	161.0650	133.0406
Daughter ions m/z (ra %)	160.86 (22)	160.00 (49)	82.79 (12)
	159.86 (15)	132.91 (92)	74.90 (8)
	132.72 (13)	74.90 (9)	

Table 3. HRMS data of molecular ions for the target compounds.

Compound	Observed m/z (% ra)	Elemental Composition (estimated m/z)
3a	162.0433 (100)	$C_8H_6O_2N_2$ (162.1454)
3b	162.0432 (100)	$C_8H_6O_2N_2$ (162.1454)
4a	203.9982 (100)	$C_9H_4O_2N_2S$ (204.2046)
4b	203.9981 (100)	$C_9H_4O_2N_2S$ (204.2046)



Scheme 2. Fragmentation pattern of **3a-b** and **4a-b**, pathways investigated and confirmed by • linked scans and ♦ high resolution techniques.

Experimental

General remarks

The nitrophthalimide substrates **2a-b** were obtained using previously reported procedures (8). The intermediates and the final products were characterized by common spectral data: IR spectra were recorded on a Nicolet FTIR-SSX spectrometer. The ^{1}H and ^{13}C NMR data were measured on a Varian Gemini 300 spectrometer at 300 MHz and 75 MHz respectively employing $CDCl_3$ or DMSO-d_6 as the solvent; the chemical shifts are expressed in ppm, using TMS (δ = 0) as an internal standard. Splitting patterns are indicated as: s singlet, d doublet, bs broad singlet , dd double-double, t triple, m multiplet. Mass spectra measurements were taken with a JEOL (Peabody, MA, USA) JMS-SX 102 and JEOL JMS-AX 505 HA mass spectrometers, using positive ion mode techniques. Thiophosgene was acquired from Aldrich Chemical Co., Inc., Cat. No. 11,515-0 and directly employed without previous treatment. Thin layer chromatography (*tlc*) were performed using Merck precoated *tlc* plates silica gel 60 F 254, 0.25 mm. Flash column chromatography (*fcc*) was employed using silica gel 60. The melting points were determined with a Fisher Johns apparatus and are uncorrected.

Aminophthalimides

A solution of (3- or 4-) nitrophthalimide (0.50 g) (**2a-b**) in methanol (50 mL) was treated to hydrogenation at atmospheric pressure, using Pd/C (0.05 g) as the catalyst. The advancement of the reaction was performed by means of *tlc*. Thereafter the reaction mixtures were filtered on celite to eliminate the catalyst; the resulting dissolution was evaporated under vacuum, being obtained in either case a solid, finally purified by *fcc* (*n*-hexane/EtOAc 2:1).

3-aminophthalimide, (**3a**): (0.318 g) 75.8 %; green yellow solid; mp. 235 °C; **IR** ν cm^{-1} (KBr): 3493, 3354, 3185, 2756, 1753, 1714, 1629, 1595, 1479, 1311, 1058, 746, 661; **^{1}H NMR** (300 MHz) $CDCl_3$/TMS δ (ppm): 10.85 (s, 1H, NH), 7.41 (dd, 1H, J *ortho* = 12.6 Hz / J *ortho* =10.6 Hz, H-5), 6.94 (dd, 1H, J *ortho* = 12.6 Hz / J *meta* = 1.1 Hz, H-6), 6.89 (dd, 1H, J *ortho* = 10.6 Hz / J *meta* = 1.1 Hz, H-4), 6.35 (s, 2H, NH_2); **^{13}C NMR** (75 MHz) $CDCl_3$/TMS δ (ppm): 133.1 (C-1), 110.5 (C-2), 146.0 (C-3), 110.2 (C-4), 120.4 (C-5), 133.1 (C-6), 169.0 (C_7=O), 170.7 (C_8 =O).

4-aminophthalimide, (**3b**): (0.265 g) 63.1 %; yellow solid; mp. 248-251 °C; **IR** ν cm^{-1} (KBr): 3444, 3362, 3242, 1762, 1714, 1616, 1506, 1390; **^{1}H NMR** (300 MHz) DMSO-d_6/TMS δ (ppm): 10.72 (s, 1H, NH), 7.43 (d, 1H, J *ortho* = 12.2 Hz, H-6), 6.86 (d, 1H, J *meta* = 2.8 Hz, H-3), 6.79 (dd, 1H, J *ortho* = 12.2 Hz / J *meta* = 2.8 Hz, H-5), 6.40 (s, 2H, NH_2); **^{13}C NMR** (75 MHz) DMSO-d_6/TMS δ (ppm): 135.4 (C-1), 117.8 (C-2), 106.6 (C-3), 154.8 (C-4), 116.8 (C-5), 124.6 (C-6), 169.6 (C_7 =O), 169.3 (C_8 =O).

Phthalimide Isothiocyanates.

To a mixture of 0.1 g (0.6 mmol) of **3a** or **3b**, 2 mL of HCl 10% and 10 mL of concentrated AcOH, was added slowly a solution of thiophosgene 0.4 % in $CHCl_3$ (15 mL for **4a** and 5 mL for **4b**); then, they were gently refluxed, monitoring the reaction by *tlc* until the disappearance of the substrate, the ones which revealed on the UV light (365 nm) and with $CeSO_4$ showed an obscure stain. The final reaction mixture was washed with $NaHCO_3$ 5 % to eliminate the remaining thiophosgene, then it was dried with anh. Na_2SO_4 and carried out to dryness. Finally **4a** and **4b** were purified by *fcc*, using *n*-Hexane/EtOAc 2:1 as the eluent.

Phthalimide 3-isothiocyanate, (**4a**): (0.067 g) 53.2 %; yellow-white solid; mp. 235 °C; **IR** ν cm^{-1} (KBr): 3207, 3083, 2058, 2004, 1770, 1740, 1699, 1603, 1476, 1378, 1306, 1068, 820, 749; **^{1}H NMR** (300 MHz) $CDCl_3$/TMS δ (ppm): 10.1 (s, 1H, NH), 7.73 (dd, 1H, J *ortho* = 11.1 Hz / J *ortho* = 10.0 Hz, H-5), 7.48 (dd, 1H, J *ortho* = 11.1 Hz / J *meta* = 2.0 Hz, H-4), 7.47 (dd, 1H, J *ortho* = 10.0 Hz / J *meta* = 2.0 Hz, H-6); **^{13}C NMR** (75 MHz) $CDCl_3$ + DMSO-d_6/TMS δ (ppm): 135.0 (C-1), 134.1 (C-2), 135.0 (C-3), 121.1 (C-4), 130.8 (C-5), 134.8 (C-6), 166.5 (C_7=O), 167.4 (C_8=O), 139.5 (N=**C**=S).

Phthalimide 4-isothiocyanate, (**4b**): (0.058 g) 46.0 %; yellow-orange solid; mp. 220 °C; **IR** ν cm^{-1} (KBr): 3202, 3086, 2056, 1766, 1730, 1698, 1613, 1358, 1300; **^{1}H NMR** (300 MHz) DMSO-d_6/TMS δ (ppm): 10.2 (s, 1H, NH), 7.86 (d, 1H, J *ortho* =11.2 Hz, H-6), 7.67 (d, 1H, J *meta* = 2.5 Hz, H-3), 7.55 (dd, 1H, J *ortho* = 12.0 Hz / J *meta* = 2.5 Hz, H-5); **^{13}C NMR** (75 MHz) DMSO-d_6/TMS δ (ppm): 132.0 (C-1), 134.3 (C-2), 124.0 (C-3), 134.5 (C-4), 128.0 (C-5), 129.0 (C-6), 167.0 (C_7=O), 166.0 (C_8=O), 139.5 (N=**C**=S).

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