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Unprecedented spirocyclization of 3-methyleneindoline-2-thiones during hydrolysis of the phytoalexin cyclobrassinin

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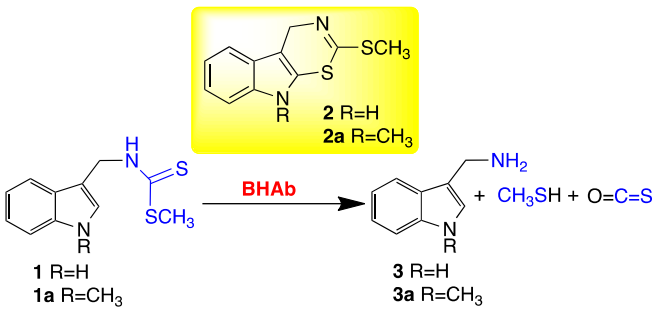
ABSTRACT

The phytoalexin cyclobrassinin is a plant defense that has additional importance since it inhibits brassinin hydrolase, a phytoalexin detoxifying enzyme produced by the plant pathogen *Alternaria brassicicola*. Hence, the 1,3-thiazino[6,5-*b*]indole scaffold of cyclobrassinin has great application as a lead structure to design potential inhibitors of brassinin detoxification. For this reason, it is necessary to determine whether *A. brassicicola* is able to transform cyclobrassinin. During this work new reactions of 1,3-thiazino[6,5-*b*]indoles and indoline-2-thiones and their unique [4+2] cycloaddition products were discovered and characterized.

Brassinin (**1**) and cyclobrassinin (**2**) are phytoalexins produced by cruciferous plants (e.g., canola, mustard, rutabaga, and cabbage) in response to stress caused by microbial pathogens and abiotic factors.[1] In addition to the traditional defense role and antimicrobial activity of phytoalexins, cyclobrassinin (**2**) has been shown to competitively inhibit the fungal enzyme brassinin hydrolase (BHAb) from *Alternaria brassicicola*,[2] which catalyzes brassinin (**1**) detoxification to 3-indolylmethanamine (**3**, Scheme 1). Enzyme inhibitors of phytoalexin detoxifying reactions (paldoxins) have been designed for treatment of fungal diseases of cruciferous crops caused by pathogens such as *A. brassicicola*.[3] For this reason, the 1,3-thiazino[6,5-*b*]indole scaffold of **2** is an obvious lead structure to design inhibitors of brassinin (**1**) detoxification. However, before embarking in such a study, it is necessary to investigate whether cyclobrassinin (**2**) is transformed by *A. brassicicola*. During this investigation, the chemistry of 1,3-thiazino[6,5-*b*]indoles and indoline-2-thiones revealed unique products that are disclosed here for the first time.

Experiments using cultures of *A. brassicicola* incubated with cyclobrassinin (**2**) indicated a very fast biotransformation, yielding a main product that could not be obtained in sufficient amount for complete spectroscopic and MS characterization. For example, the ^{1}H NMR spectrum of this product (CD_3OD) displayed all proton signals corresponding to cyclobrassinin (**2**), with slightly different chemical shifts, but no reasonable MS data could be obtained using any of the various ionization techniques available to us (EI, CI, ESI, FABS). Consideration of the ^{1}H NMR signals suggested that the product resulted from enzymatic hydrolysis of cyclobrassinin (**2**). Hence, in an attempt to obtain larger amounts of this product, the chemical hydrolysis of cyclobrassinin (**2**) was carried out under various conditions. Eventually, a major product plus a number of undetermined products were obtained under acidic conditions. The structure of the major product was tentatively assigned as **4**, however its recovery after work up (neutralization and extraction) was rather low (<15%). To increase the yield of **4**, the reaction mixture was concentrated to dryness and the residue was rinsed with EtOAc and/or MeOH, but none of these solutions contained a



Scheme 1. Detoxification of the phytoalexin brassinin (**1**) catalyzed by brassinin hydrolase (BHAb) and inhibited by cyclobrassinin (**2**).

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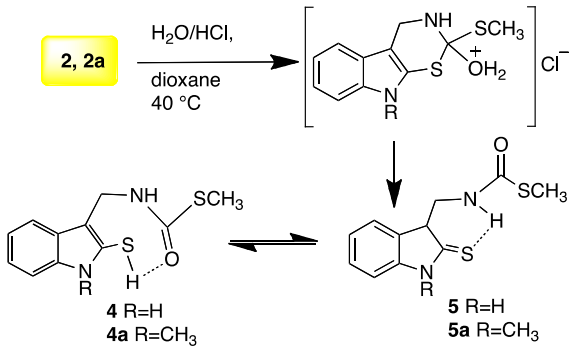

Scheme 2. Hydrolysis products of cyclobrassinin (**2**) and methylcyclobrassinin (**2a**), proposed intermediates in square brackets; only tautomers **4** and **4a** are present in DMSO-d_6 solution.

significant amount of **4**; most of the residue remained insoluble in any of these solvents (Scheme 2). Eventually, DMSO was used to dissolve this residue, which after acquisition of ^{1}H and ^{13}C NMR and HMBC spectroscopic data was assigned to be *S*-methyl [(2-sulfanyl-1*H*-indolyl-3)methyl]carbamothioate (**4**). The presence of tautomer **5** in DMSO-d_6 solution was ruled out from analysis of the NMR spectroscopic data. The low solubility of **4** in organic solvents, including polar solvents such as MeOH and EtOH, was somewhat puzzling. Reasoning that hydrolysis of 1-methylcyclobrassinin (**2a**) might yield a product with higher solubility in organic solvents, **2a** was prepared and subjected to similar reaction conditions to yield **4a** as the major product, together with a few minor products (Scheme 2). As in the case of **4**, **4a** was soluble in DMSO while two minor products were soluble in CH_2Cl_2. The presence of tautomer **5a** was ruled out based on its ^{1}H and ^{13}C NMR spectral data.

Unexpectedly, spectroscopic characterization of the minor products of hydrolyses of **2** and **2a** suggested novel spirocyclic indoline-2-thiones, based on the following analysis. One of the minor products (<5%, as described in Supplementary data) of acidic hydrolysis of cyclobrassinin (**2**) displayed a molecular formula of $C_{18}H_{14}N_2S_2$, as suggested by HRMS-EI and corroborated by NMR spectroscopic data. The ^{1}H NMR spectrum displayed signals for 14 protons, of which eight were aromatic (δ_H 7.0–7.5), two were D_2O exchangeable (δ_H 9.51 and 7.94) and four were from methylene groups (δ_H 3.65, d, J = 16 Hz, 1H; 2.83, dd, J = 16, 2 Hz, 1H; 3.87, d, J = 13 Hz, 1H; 2.64, dd, J = 13, 2 Hz, 1H). The ^{13}C NMR spectrum displayed signals for 18 carbons, of which a carbon signal at δ_C 210.8 was assigned to a thiocarbonyl group. Detailed analysis of the HMQC and HMBC data indicated that the proton at δ_H 3.65 correlated to the C=S at δ_C 210.8, and both methylene protons at δ_H 3.65 and 2.83 correlated with a quaternary carbon at δ_C 106.2. This structural analysis suggested structure **7**, a dimer of 3-methyleneindoline-2-thione (**6**), a new structure resembling compounds resulting from condensation of indoline-2-thiones with aldehydes, followed by [4+2] cycloadditions.[4–6] Another product obtained together with **7** could not be purified.[7] Similarly, hydrolysis of 1-methylcyclobrassinin (**2a**) yielded several minor products, two of which were obtained in sufficient amounts to purify by preparative

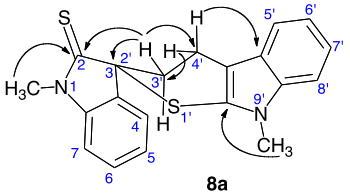

Figure 1. Selected HMBC correlations of compound **8a**.

TLC (<10%, as described in Supplementary data). These products were structural isomers, as suggested by their identical molecular formulas ($C_{20}H_{18}N_2S_2$) deduced from HRMS (EI and ESI) and NMR spectral data. The molecular formulas indicated that each isomer had two additional methyl groups relative to compound **7**. Similar to **7**, the ^{1}H NMR of one of the products (**7a**) displayed two aromatic spin systems of four protons each, two spin systems of methylene protons (δ_H 3.88, d, J = 13 Hz, 1H; 2.58, dd, J = 13, 2 Hz, 1H; 3.64, d, J = 16 Hz, 1H; 2.77, dd, J = 16, 2 Hz, 1H) and three methyl singlets, two of which were attributable to *N*-methyl groups and another to an S-methyl. The ^{1}H NMR of the other minor compound (**8a**) also displayed two independent aromatic spin systems of four protons each, and a spin system of two methylene protons, clearly different from those observed in **7a** (δ_H 3.33, ddd, J = 17, 12, 6 Hz and 3.10, ddd, J = 17, 12, 6 Hz; 2.85, ddd, J = 14, 12, 6 Hz and 2.04, ddd, J = 14, 12, 6 Hz). As indicated by their coupling constants, the methylene protons of this product were adjacent to each other, suggesting structure **8a**, a structural isomer of **7a**. Detailed analysis of the HMQC and HMBC data of each compound was consistent with these structural assignments. For example, the low field carbon of **8a** at δ_C 203.3 correlated to *N*-methyl protons at δ_H 3.74, thus allowing its assignment as C-2 of an indoline-2-thione ring (Fig. 1). The methylene proton at δ_H 3.33 correlated with carbon at δ_C 104.9 and a quaternary sp^3 carbon at δ_C 62.5, as summarized in Figure 1. Importantly, while structures **7** and **7a** could be rationalized as products of [4+2] cycloadditions, that is, Diels–Alder reactions of 3-methyleneindoline-2-thiones,[4] the formation of **8a** appeared to be unprecedented. For this reason, we investigated the preparation of these spirocyclic indoline-2-thiones using another route, as follows.

Although 3-methyleneindolin-2-one has been prepared in different occasions,[8–10] preparation of 3-methyleneindoline-2-thione (**6**) has not been reported to date. Considering the substantially higher acidity of indoline-2-thiones versus indolin-2-ones,[11] we anticipated that **6a** could be formed by condensation of 1-methylindoline-2-thione (**9a**) with formaldehyde, by analogy to the condensation of indoline-2-thione (**9**) with benzaldehyde.[4] We were delighted to find that condensation of 1-methylindoline-2-thione (**9a**) with formaldehyde proceeded under acid catalysis and was followed by cycloaddition to afford compounds **7a** and **8a** in a 1:1 ratio (32% yield, Supplementary data). Similar conditions applied to thione **9b** provided compounds **7b** and **8b** in a 1.2:1 ratio (46% yield). However, several attempts to obtain equivalent products from condensation of **9** with formaldehyde led to multiple products in which **7** was a minor component, as determined by HPLC analysis of the reaction mixture.

To the best of our knowledge, neither the reaction of indoline-2-thiones **9a** or **9b** with formaldehyde, nor the cycloaddition compounds **7**, **7a**, **7b**, **8a** and **8b** have been previously reported. The formation of **8a** or **8b** resulting from [4+2] intermolecular cycloadditions of 3-methyleneindoline-2-thiones **6a** or **6b** (acting as diene and dienophile, B, Scheme 3) is unprecedented. By contrast, as mentioned above, the formation of **7a** and **7b** also resulting from [4+2] intermolecular cycloadditions (A, Scheme 3) has literature precedent.[4] It was particularly surprising to find that the amounts of **7a** and **7b** versus **8a** and **8b** cycloaddition products were similar. In addition, it was established that purified products **7b** or **8b** did not interconvert under conditions identical to those used in the condensation reactions.

Due to the unprecedented nature of some of the products of these cycloaddition reactions, there was a need to further confirm the molecular structures of **8a** or **8b**. Crystallization of **8b** by slow evaporation of a hexane–Et_2O solution yielded crystals from which a single crystal structure that confirmed the NMR structural assignments described above was obtained by X-ray crystallography (Fig. 2).