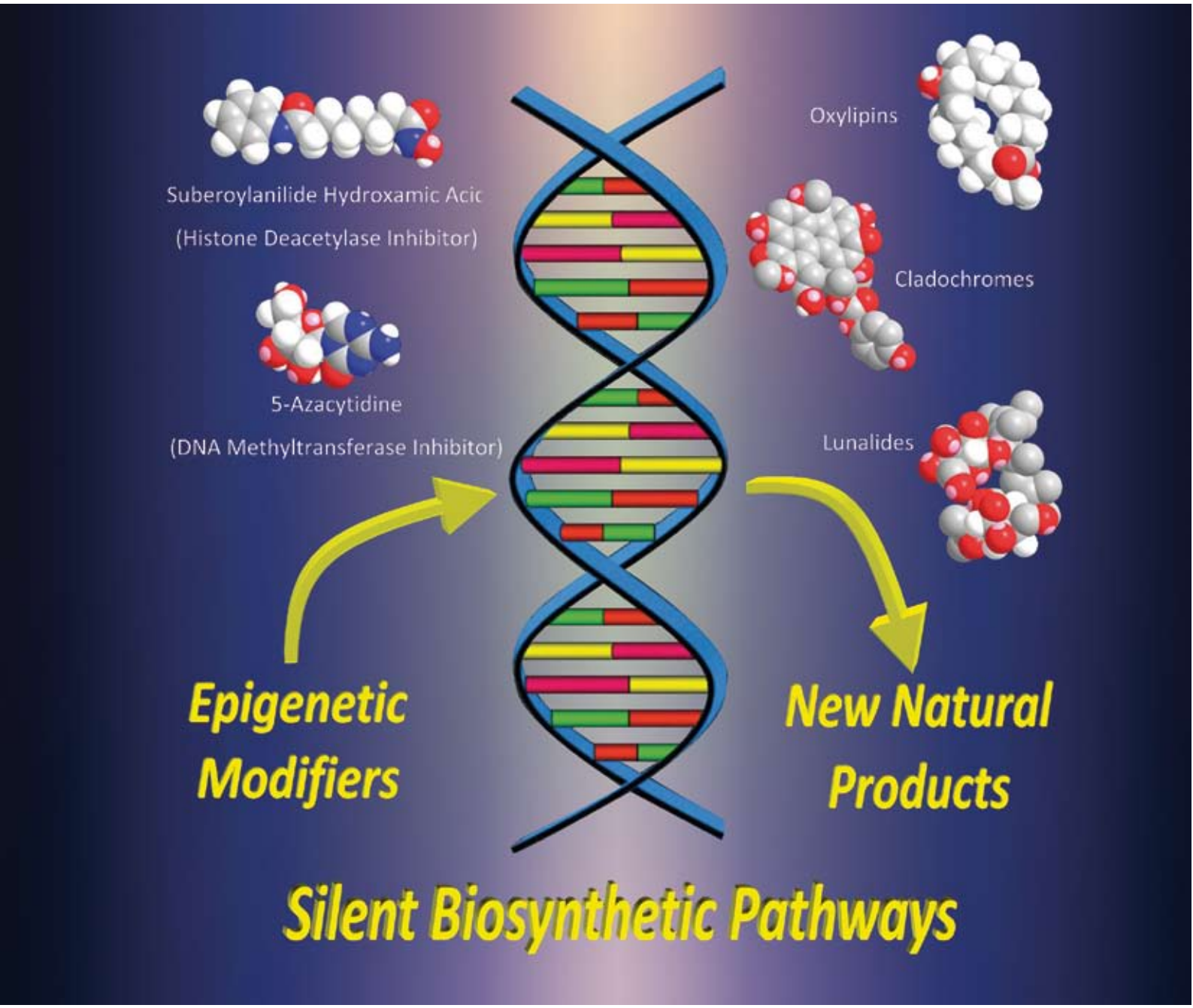
Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 6 | Number 11 | 7 June 2008 | Pages 1857–2020



ISSN 1477-0520

RSC Publishing

Epigenetic remodeling of the fungal secondary metabolome†

Russell B. Williams, Jon C. Henrikson, Ashley R. Hoover, Andrlynn E. Lee and Robert H. Cichewicz*‡

Received 19th March 2008, Accepted 4th April 2008
First published as an Advance Article on the web 14th April 2008
DOI: 10.1039/b804701d

Fungi treated with DNA methyltransferase and histone deacetylase inhibitors exhibited natural product profiles with enhanced chemical diversity demonstrating that small-molecule epigenetic modifiers are effective tools for rationally controlling the native expression of fungal biosynthetic pathways and generating new biomolecules.

Fungi are biosynthetically talented organisms capable of producing a wide range of chemically diverse and biologically intriguing small molecules. The majority of scientific interests in fungal natural products have centered on their pharmaceutical applications, roles as mycotoxins, and multifarious ecological functions. Unfortunately, typical fungal fermentation methods such as axenic shake or static cultures on artificially defined media are poor surrogates for mimicking an organism's native habitat. The consequence of these practices is that only a subset of the biosynthetic pathways which encode for secondary metabolite production in fungi are ever expressed, thus limiting prospects for realizing the complete drug discovery potential of these organisms. Moreover, without securing the full range of 'silent'[1] secondary metabolites, opportunities for testing hypothesis-based inquiries regarding their native functions (antibiosis, intra- and inter-species communication, and modulation of diverse biotic responses) remain unattainable. The magnitude of this problem has become markedly apparent with the advent of modern genomic technologies. With the exception of yeast, the number of putative natural product biosynthetic pathways in fungi exceeds the sum total of natural products observed under laboratory culture conditions. Pertinent examples highlighting this phenomenon are found among *Aspergillus* spp.,[2] *Neurospora crassa*,[3] and *Magnaporthe grisea*.[4]

Methods developed for exploring the products of silent secondary metabolic pathways in microorganisms fall into two broad categories:[5] molecular-based techniques and cultivation-dependent approaches. Despite their incredible promise for providing outstanding access to and control over silent biosynthetic pathways, molecular-based methods utilizing heterologous expression systems are limited by problems such as locating and cloning genes, difficulties with gene transformation and inactivation, and host incompatibilities. Alternatively, a variety of cultivation-dependent procedures in which biotic and abiotic culture parameters are manipulated have likewise been proposed. While this methodology has been a mainstay of microbial natural products for decades, the concept was more recently formalized by Zeeck and colleagues[6] and has since been applied by several groups.[7] Unfortunately, this protractive strategy is labor intensive, lacks predictable outcomes, and inflates the workload of natural products screening programs. These difficulties are further compounded by strain-specific variation in the quantity of metabolite production as well as the seemingly capricious behavior of fungi to alter metabolite profiles when re-cultured. Consequently, an alternative methodology that is universally applicable for rationally inducing the expression of silent natural product biosynthetic pathways in fungi is needed.

The discovery of the putative nuclear transcriptional regulator LaeA, which controls secondary metabolite production in *Aspergillus*,[8] has inspired our group to examine global mechanisms by which fungi restrictively manage the production of natural products under laboratory culture conditions. Published fungal genomes demonstrate a propensity for the positioning of many putative natural product biosynthetic gene clusters in the distal regions of chromosomes.[9] Importantly, these portions of fungal genomes are noted to exist in a heterochromatin state whose constitutive genes are often transcriptionally controlled by epigenetic regulation such as histone deacetylation and DNA methylation. Recently, Keller and colleagues have demonstrated that disruption of histone deacetylase activity (Δhda) in *Aspergillus nidulans* led to the transcriptional activation of gene clusters for the production of sterigmatocystin and penicillin.[9] Moreover, extension of these observations to growing cultures of *Alternaria alternate* and *Penicillium expansum* suggested that inhibition of histone deacetylase activity could positively modulate secondary metabolite production. Based on these observations, we hypothesized that small-molecule epigenetic modifiers could be rationally employed for accessing silent natural product pathways and enhancing the native production of fungal secondary metabolites. The development of such an approach would present significant advantages to the natural products research community for controlling the expression of latent biosynthetic pathways and creating new opportunities for novel small-molecule discoveries.

Our initial experiments testing this approach consisted of subjecting a diverse panel of twelve fungi to treatment with a focused library of DNA methyltransferase and histone deacetylase inhibitors in dose dilution series (0.1 μM to 10 mM).† Comparative profiling by HPLC, MS, ^{1}H-NMR and TLC demonstrated eleven of the fungi were responsive to one or more epigenetic treatments based on the production of new natural products and/or enhanced accumulation of constitutive secondary metabolites. Furthermore, exposure of selected *Aspergillus*, *Cladosporium*, *Clonostachys*, *Diatrype*, *Penicillium*, and other fungal strains to multiple media types confirmed that many of the new natural products were exclusively obtained following the use of epigenetic modifying treatments. Cultures treated with amphotericin B, cycloheximide, and 5-fluorouracil did not produce new or enhanced secondary

Natural Products Discovery Group, Department of Chemistry and Biochemistry, 620 Parrington Oval, Room 208, University of Oklahoma, Norman, Oklahoma, 73019-3032, USA. E-mail: rhcichewicz@ou.edu; Fax: +1 (405) 325-6111; Tel: +1 (405) 325-6969

† Electronic supplementary information (ESI) available: Experimental details, structure characterization, additional results with figures and tables. See DOI: 10.1039/b804701d

‡ Graduate Program in Ecology and Evolutionary Biology.

metabolite profiles indicating that epigenetic modifiers impacted fungi in a manner that was functionally distinct from that of a general cytotoxic response. Interestingly, a combination treatment, composed of a DNA methyltransferase inhibitor and histone deacetylase inhibitor, was tested and determined to be only modestly effective due to significant growth restriction and/or generation of metabolite profiles dominated by the effects of a single component in the mixture (*e.g.* effects of individual compound treatments were not additive as a mixture).

In order to probe the nature of metabolic remodeling induced by epigenetic modifier treatment, two fungi were selected for scale-up studies. The first isolate was obtained from a tidal pool along the coastline of Casco Bay, Portland, Maine, USA, and identified as *Cladosporium cladosporioides* by analysis of a 300 base pair sequence of the D2 region of the 26S large ribosomal subunit rRNA gene and morphological considerations.† This culture exhibited divergent responses to the small-molecule epigenetic modifiers 5-azacytidine and suberoylanilide hydroxamic acid which led to dramatic restructuring of its secondary metabolome with both treatments (Fig. 1A,C). Treatment of *C. cladosporioides* with 5-azacytidine elicited the *de novo* production of several oxylipins, three of which were characterized by NMR and MS analysis† as (9*Z*,12*Z*)-11-hydroxyoctadeca-9,12-dienoic acid (**1**), its methyl ester (**2**), and glycerol conjugate (**3**) in substantial yields (Fig. 1B). The production of these compounds is of considerable interest since these types of metabolites are widely recognized for their important roles as intra- and inter-species cell signaling molecules. In contrast, suberoylanilide hydroxamic acid induced the production of a complex series of perylenequinones, two of which were characterized as new metabolites, cladochromes F (**4**) and G (**5**), along with four known cladochromes A (**6**), B (**7**), D (**8**), and E (**9**) and calphostin B (**10**) (Fig. 1D. and see ESI†). This is remarkable since this is the first reported co-occurrence of such an extensive range of cladochrome-calphostin metabolites from a single source. Moreover, the identification of **6** and **7** under epigenetic stimulation is significant since these compounds were first reported as the unique products of *Cladosporium* infection of *Cucumis sativus* seedlings, yet could not be obtained from an extensive number of mono-culture fermentations.[10] While it was originally speculated that **6** and **7** might be the products of mixed *Cladosporium–Cucumis* biosynthesis, their select production in suberoylanilide hydroxamic acid treated *Cladosporium* suggests that their biogenesis is normally tightly regulated and sensitive to some yet undefined host-specific signaling event.

The second fungal isolate was obtained from the foregut of a fifth instar luna moth (*Actias luna*; Saturniidae) larva that was cultured on an exclusive diet of sweet gum (*Liquidambar styraciflua* L.; Hamamelidaceae) leaves. Initial characterization of the fungus by analysis of the 26S rRNA gene gave a 93% sequence homology to *Diatrype disciformis.* While control cultures of this *Diatrype* sp. were relatively void of any secondary metabolites, addition of 5-azacytidine triggered a significant change in the organism's metabolic profile (Fig. 2A), resulting in the production of two new polyketides, lunalides A (**11**) and B (**12**) (Fig. 2B). It is interesting to note that one other non-epigenetic culture treatment, elicitation with *E. coli*,† resulted in the biosynthesis of **11** and **12** which were otherwise repressed under axenic culture conditions. The results suggest that their production is under specific control of a unique environmental cue.

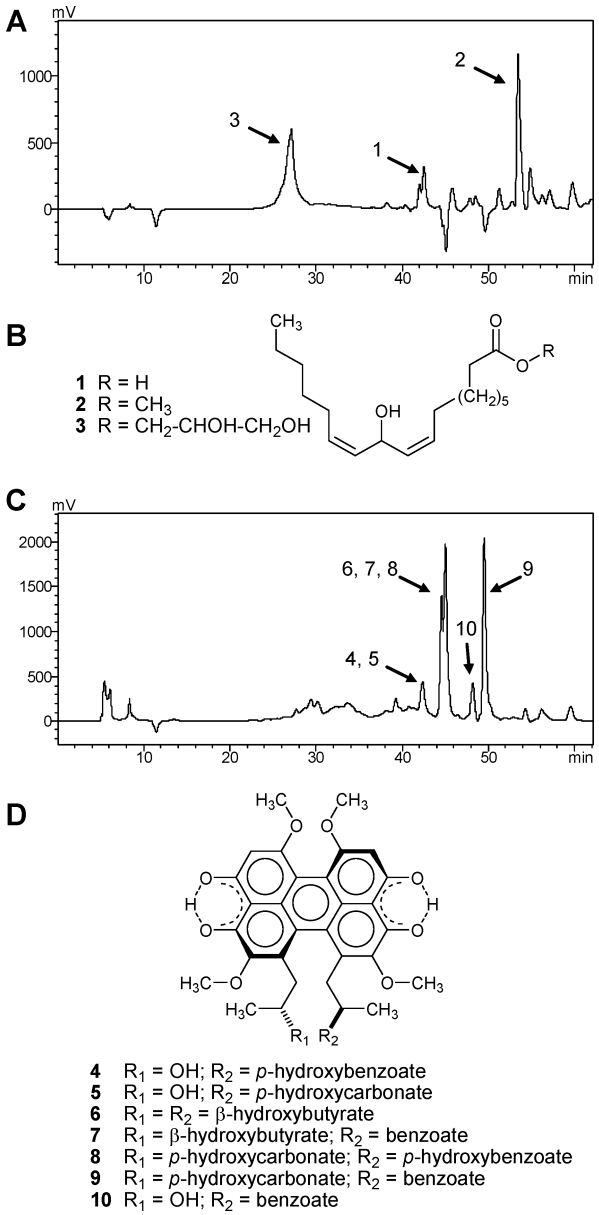


Fig. 1 Difference chromatograms and structures of *C. cladosporioides* natural products following treatment with epigenetic modifiers. Ten cultures within each treatment group were pooled and the total organic extract separated by C_{18} HPLC with UV detection (UV trace at 210 nm). Difference chromatograms were generated by subtracting the chromatogram of the untreated control from the treatment groups. Peaks phasing upward represent metabolites expressed only upon epigenetic treatment or produced at enhanced concentrations. (A) Difference chromatogram following 5-azacytidine administration yielding oxylipins **1–3** (B). (C) Difference chromatogram demonstrating the effect of suberoylanilide hydroxamic acid on secondary metabolite expression leading to the production of perylenequinones **4–10** (D). Compounds were characterized by HRESIMS, NMR, and CD and/or by comparison to values published in the literature.†

The success of inducing the production of new natural products from fungi by administering small-molecule epigenetic modifiers indicates that this technique is a very promising and rational approach for the native expression of silent biosynthetic pathways.

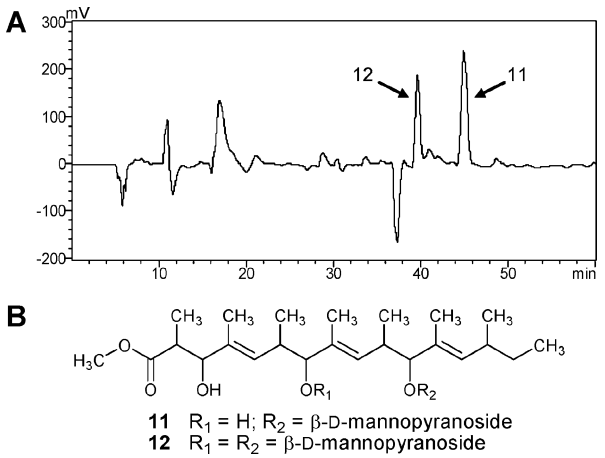


Fig. 2 (A) Difference chromatogram (UV trace at 210 nm) for *Diatrype* sp. treated with 5-azacytidine. (B) New glycosylated polyketides **11** and **12** (lunalides A and B, respectively) were produced following treatment with the DNA methyltransferase inhibitor. Compounds were characterized by HRESIMS and NMR.†

This method has several significant benefits compared to currently available molecular or culture-dependent techniques. First and foremost, it provides a needed tool for rapidly accessing potential pools of cryptic fungal natural products in their native hosts. Second, this methodology can be readily implemented in most labs without extensive retooling, giving it a wide scope of utilization. Third, this approach will significantly lessen the cost and effort of acquiring the products of silent secondary metabolic pathways since fungi do not need to be pre-screened using a multitude of culture conditions.

Acknowledgements

This work was supported by The University of Oklahoma, College of Arts and Sciences, The University of Oklahoma Department of Chemistry and Biochemistry, and the American Cancer Society (IRG-05-066-01). *Liquidambar styraciflua* leaves were kindly provided by K. Hobson and C. Van Buskirk. We thank L. Clifford, W. Gutekunst, M. Joyner, and R. Matheke for their helpful comments during the preparation of the manuscript.

Notes and references

1 S. Bergmann, J. Schumann, K. Scherlach, C. Lange, A. A. Brakhage and C. Hertweck, *Nat. Chem. Biol.*, 2007, **3**, 213.
2 W. C. Nierman, A. Pain, M. J. Anderson, J. R. Wortman, H. S. Kim, J. Arroyo, M. Berriman, K. Abe, D. B. Archer, C. Bermejo, J. Bennett, P. Bowyer, D. Chen, M. Collins, R. Coulsen, R. Davies, P. S. Dyer, M. Farman, N. Fedorova and N. Fedorova, *Nature*, 2005, **438**, 1151.
3 J. E. Galagan, S. E. Calvo, K. A. Borkovich, E. U. Selker, N. D. Read, D. Jaffe, W. FitzHugh, L.-J. Ma, S. Smirnov, S. Purcell, B. Rehman, T. Elkins, R. Engels, S. Wang, C. B. Nielsen, J. Butler, M. Endrizzi, D. Qui, P. Ianakiev, D. Bell-Pedersen, M. A. Nelson, M. Werner-Washburne, C. P. Selitrennikoff, J. A. Kinsey, E. L. Braun, A. Zelter, U. Schulte, G. O. Kothe, G. Jedd, W. Mewes, C. Staben, E. Marcotte, D. Greenberg, A. Roy, K. Foley, J. Naylor, N. Stange-Thomann, R. Barrett, S. Gnerre, M. Kamal, M. Kamvysselis, E. Mauceli, C. Bielke, S. Rudd, D. Frishman, S. Krystofova, C. Rasmussen, R. L. Metzenberg, D. D. Perkins, S. Kroken, C. Cogoni, G. Macino, D. Catcheside, W. Li, R. J. Pratt, S. A. Osmani, C. P. C. DeSouza, L. Glass, M. J. Orbach, J. A. Berglund, R. Voelker, O. Yarden, M. Plamann, S. Seiler, J. Dunlap, A. Radford, R. Aramayo, D. O. Natvig, L. A. Alex, G. Mannhaupt, D. J. Ebbole, M. Freitag, I. Paulsen, M. S. Sachs, E. S. Lander, C. Nusbaum and B. Birren, *Nature*, 2003, **422**, 859.
4 R. A. Dean, N. J. Talbot, D. J. Ebbole, M. L. Farman, T. K. Mitchell, M. J. Orbach, M. Thon, R. Kulkarni, J.-R. Xu, H. Pan, N. D. Read, Y.-H. Lee, I. Carbone, D. Brown, Y. Y. Oh, N. Donofrio, J. S. Jeong, D. M. Soanes, S. Djonovic, E. Kolomiets, C. Rehmeyer, W. Li, M. Harding, S. Kim, M.-H. Lebrun, H. Bohnert, S. Coughlan, J. Butler, S. Calvo, L.-J. Ma, R. Nicol, S. Purcell, C. Nusbaum, J. E. Galagan and B. W. Birren, *Nature*, 2005, **434**, 980.
5 H. Gross, *Appl. Microbiol. Biotechnol.*, 2007, **75**, 267.
6 H. B. Bode, B. Bethe, R. Höfs and A. Zeeck, *ChemBioChem*, 2002, **3**, 619.
7 P. A. Paranagama, E. M. K. Wijeratne and A. A. L. Gunatilaka, *J. Nat. Prod.*, 2007, **70**, 1939; K. Scherlach and C. Hertweck, *Org. Biomol. Chem.*, 2006, **4**, 3517; T. Amagata, M. Tanaka, T. Yamada, M. Doi, K. Minoura, H. Ohishi, T. Yamori and A. Numata, *J. Nat. Prod.*, 2007, **70**, 1731.
8 R. M. Perrin, N. D. Fedorova, J. W. Bok, R. A. Cramer, J. R. Wortman, H. S. Kim, W. C. Nierman and N. P. Keller, *PLoS Pathog.*, 2007, **3**, 508.
9 E. K. Shwab, J. W. Bok, M. Tribus, J. Galehr, S. Graessle and N. P. Keller, *Eukaryotic Cell*, 2007, **6**, 1656.
10 J. C. Overeem, A. K. Sijpesteijn and A. Fuchs, *Phytochemistry*, 1967, **6**, 99; A. Arnone, G. Assante, V. Di Modugno, L. Merlini and G. Nasini, *Phytochemistry*, 1988, **27**, 1675.