A chemical epigenetics approach for engineering the *in situ* biosynthesis of a cryptic natural product from *Aspergillus niger*

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A new fungal metabolite, nygerone A (1), featuring a unique 1-phenylpyridin-4(1*H*)-one core that had previously not been reported from any natural source, has been obtained from *Aspergillus niger* using a chemical epigenetics methodology.

Transcriptional suppression of polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS), and hybrid PKS-NRPS (HPN) genes clusters has thwarted the efforts of natural products chemists to fully explore the secondary metabolomes of fungi. The full impact of the gene cluster silencing problem was recently illustrated with the soil-dwelling fungus, *Aspergillus niger*, showing that fewer than 30% of its 31 PKS, 15 NRPS, and 9 HPN biosynthetic gene clusters were transcribed under a variety of *in vitro* culture conditions.[1] The pervasive nature of this problem has led our group to explore the mechanisms by which fungi suppress biosynthetic gene cluster transcription in order to develop new methodologies for inducing the expression of silent PKS, NRPS, and HPN genes *in situ.* It has been hypothesized that epigenetic processes play important regulatory roles in PKS, NRPS, and HPN gene cluster silencing and that manipulation of these pathways would result in the *de novo* biosynthesis of cryptic fungal secondary metabolites.[2] Using a chemical biology-based approach called chemical epigenetics, we have shown that the selective manipulation of epigenetic targets using small molecule inhibitors of histone deacetylase and DNA methyltransferase activities led to the enhanced expression of PKS, NRPS, and HPN biosynthetic pathways and production of new secondary metabolites.[1,2] Applying this method, we have initiated an ambitious project aimed at fully characterizing the complete secondary metabolome of *A. niger* in order to test how this fungus utilizes natural products throughout its life history and identify novel compounds as drug discovery leads.

Small-scale screening of a focused library of naturally occurring and synthetic histone deacetylase inhibitors demonstrated that suberoylanilide hydroxamic acid (SAHA) was capable of radically restructuring the secondary metabolome of *A. niger* ATCC 1015. A scale-up culture consisting of three Fernbach-style flasks each containing 150 mL of a semi-solid vermiculite-based medium were treated with 10 μM of SAHA, inoculated with spores and hyphae of *A. niger*, and maintained under static conditions (12 h light/12 h dark, 25 °C) for two weeks. Cultures were harvested by adding 500 mL of ethyl acetate to each flask and after 12 h of extraction, the ethyl acetate and aqueous layers were decanted and filtered, the organic layer was removed by partitioning, and the solvent evaporated yielding 195 mg of ethyl acetate soluble organic matter. The total organic extract was subjected to repeated reversed-phase preparative and semi-preparative HPLC under methanol-water and acetonitrile-water gradient conditions providing 1.9 mg of nygerone A (**1**) (Fig. 1) as an off-white to slightly yellowish amorphous solid.

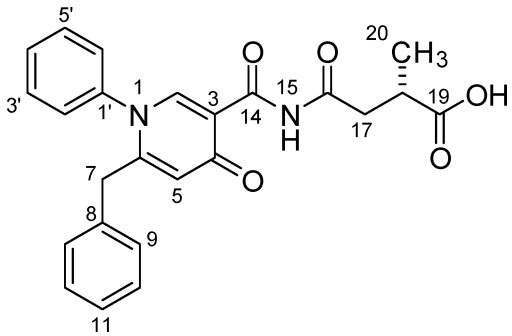

Fig. 1 A chemical epigenetics approach led to the production of nygerone A (**1**) from *Aspergillus niger via* treatment of the organism with suberoylanilide hydroxamic acid, a histone deacetylase inhibitor.

Nygerone A (**1**) was analyzed by HRESIMS providing two pseudo-molecular ions at *m/z* 419.1542 and 441.1402 corresponding to molecular formulae of $C_{24}H_{23}N_2O_5$ ($[M + H]^+$, calcd 419.1607, –6.5 mmu error) and $C_{24}H_{22}N_2NaO_5$ ($[M + Na]^+$, calcd 441.1426, –2.4 mmu error), respectively. Using these data, a chemical formula of $C_{24}H_{23}N_2O_5$ was proposed for **1** indicating that the fungal metabolite contained a total of 15 double-bond equivalents. Some minor discrepancies between the ^{1}H- and ^{13}C-NMR data for **1** (Table 1) and the MS-derived formula still required reconciliation. For example, integration of the ^{1}H NMR data collected in methanol-d_4 revealed only 20 hydrogens, but this was rationalized as evidence for the presence of two exchangeable protons in **1**. In addition, the ^{13}C NMR data provided evidence for 20 unique spins; however, four of these carbons (δ_C 127.0, 128.9, 129.0, and 130.2) exhibited significantly greater relative intensities compared to surrounding spins and thus were proposed to each represent two carbons as a result of the superimposition of chemical-shift equivalent resonances. Together, these data offered strong support for $C_{24}H_{22}N_2O_5$ as the chemical formula of **1** which permitted us to proceed with elucidating the metabolite's structure.

Further examination of the 1-D NMR results in combination with data generated from a series of 2-D NMR experiments (^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC) allowed for the establishment of four partial structures (fragments A-D) for **1** (Fig. 2). Fragment A was determined to contain five

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Table 1 ^{1}H and ^{13}C NMR data for nygerone A (**1**) in $CDCl_3$ at 400 MHz (^{1}H) and 100 MHz (^{13}C)

Position	δ_C	δ_H (mult., J in Hz)	HMBC $^{1}H \rightarrow {}^{13}C$
C-2	148.2	8.43, 1H (s)	1′, 4, 6, 14
C-3	117.2		
C-4	177.6		
C-5	122.3	6.55, 1H (s)	3, 4, 6, 7, 14 (weak)
C-6	152.0		
C-7	39.4	3.67, 2H (s)	5, 6, 8, 9/13
C-8	134.8		
C-9/13	128.9	6.77, 2H (m)	7, 11
C-10/12	129.0	7.18, 2H (m)[a]	8
C-11	127.7	7.18, 1H (m)[a]	8, 9/13
C-14	163.2		
N-15		12.83, 1H (br s)	17
C-16	173.2		
C-17	42.0	2.86, 1H (dd, 5.1, 17.2); 3.20, 1H (dd, 8.2, 17.2)	16, 18, 19, 20
C-18	35.1	3.05, 1H (m)	16, 17, 19, 20
C-19	178.9		
C-20	17.2	1.27, 3H (d, 7.0)	17, 18, 20
C-1′	140.4		
C-2′/6′	127.0	7.06, 2H (d, 7.6)	1′, 4′
C-3′/5′	130.2	7.42, 2H (t, 7.6)	1′
C-4′	130.5	7.49, 1H (t, 7.6)	2′/6′

[a] Significant overlap occurred between these two sets of resonances.

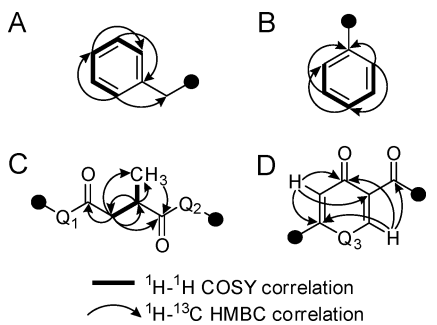

Fig. 2 NMR spectroscopic data (^{1}H-^{1}H COSY: bold bonds and ^{1}H-^{13}C HMBC: solid curved arrows) used to establish the four partial structures, fragments A-D, for **1**. Heteroatoms Q_1, Q_2, and Q_3 were subsequently assigned as N, O, and N, respectively, based on the chemical shifts of adjacent carbon atoms and ^{1}H-^{15}N HMBC correlations as illustrated in Fig. 3.

aromatic protons [δ_H 6.77 (2H), 7.18 (3H)] belonging to a mono-substituted benzyl group. This was confirmed by ^{1}H-^{13}C HMBC experiment which also gave a strong correlation extending from the aromatic spin at δ_H 6.55 to the benzylic methylene (δ_C 39.4). Similarly, fragment B (Fig. 2) contained five protons in an AA′BB′C system [δ_H 7.06 (2H), 7.42 (2H), and 7.49 (1H)] representative of a monosubstituted phenyl group. Unfortunately, ^{1}H-^{13}C HMBC couplings from the aromatic protons provided no additional evidence as to the identity of the aryl substituent of this fragment.

Analysis of the protons in fragment C by ^{1}H-^{1}H COSY indicated the presence of an A_2MX_3 spin system which was confirmed by ^{1}H-^{13}C HMBC experiment (Fig. 2). Additional $^{2\text{-}3}J_{H\text{-}C}$ correlations were also noted extending to two carbonyl resonances (δ_C 173.2 and 178.9) that were positioned at opposing ends of fragment C. Based on their chemical shifts, both carbonyl groups were presumed to be attached to heteroatoms (Q_1 and Q_2 = N and/or O, Fig. 1); however, the identities of these atoms were not determined at this juncture. Next, we turned our attention to assembling the remaining atoms that comprised fragment D which included six carbons (δ_C 117.2, 122.3, 148.2, 152.0, 163.2, and 177.6) and three heteroatoms (N and/or O). A ^{1}H-^{13}C HSQC experiment showed that two of the carbon atoms (δ_C 122.3 and 148.2) had attached protons (δ_H 8.43 and 6.55, respectively) that appeared as sharp singlet resonances. Two of the other carbons (δ_C 163.2 and 177.6) were determined to be carbonyls based on their downfield shifts and this accounted for two of the five double-bond equivalents in fragment D, as well as, two hetero- (oxygen) atoms. Similarly, the chemical shifts for the carbon atoms at δ_C 148.2 and 152.0 suggested that they were sp^2 hybridized and both attached to the remaining heteroatom, Q_3, due to their respective downfield shifts. The identity of Q_3 was determined to be a nitrogen since an oxygen at this position would have resulted in even greater deshielding of the adjacent carbon resonances (i.e. $\delta_C > 160$). Our assignment for Q_3 was further confirmed by a ^{1}H-^{15}N HMBC experiment in which the protons in fragment D (δ_H 6.55 and 8.43) exhibited $^{2\text{-}3}J_{H\text{-}N}$ correlations to a nitrogen resonating at δ_N 158.0 (Fig. 3). The assignments of the carbon atoms at δ_C 117.2 and 122.3 were now simplified since the methine carbons could not be adjacent to one another due to a lack of vicinal coupling between their attached protons. The upfield shifts of these carbons were rationalized as due to their positions α to a carbonyl, thus fragment D was comprised of a substituted γ-pyridone system.

With working structures proposed for fragments A-D, it was now possible to address the connectivities between these partial structures, as well as clarify the identities of Q_1 and Q_2. Fragment A was joined to the γ-pyridone based on long-range $^{2\text{-}3}J_{H\text{-}C}$ correlations from the benzylic methylene protons (δ_H 6.55, H-7) to carbons at δ_C 122.3 (C-5) and 152.0 (C-6) in fragment D (Fig. 3). Fragment B was also determined to be attached to fragment D as a result of a $^{3}J_{H\text{-}C}$ correlation from δ_H 8.43 (H-2) to δ_C 140.4 (C-1′). The connection established from the phenyl group through the nitrogen atom yielding a 1-phenylpyridin-4(1*H*)-one system was rather intriguing to us since this unusual substructure has not been previously reported from a natural source. With only two isolated bonds remaining to be joined, it was readily apparent that fragment D was also connected to fragment C through Q_1 or Q_2; however, no long-range coupling could be observed to verify this linkage. Despite this problem, we were able to confirm the identity of Q_1 based on 1) a $^{1}J_{H\text{-}N}$ correlation observed in $CDCl_3$ between the proton at δ_H 12.83 (1H, s) and a nitrogen at δ_N 154.8 and 2) an additional $^{3}J_{H\text{-}C}$ correlation from this proton to the methylene carbon at δ_C 42.0 (C-17). With all of the other heteroatoms accounted for in the molecular formula of **1**, Q_2 was deduced to be the last remaining oxygen atom. We proposed that the connectivity between fragments C and D occurred through the nitrogen atom, rather than the oxygen, since this mono-hydrogenated nitrogen still required one additional covalent bond in order to complete its valence shell.

During the course of our efforts to further optimize ^{1}H-^{13}C and ^{1}H-^{15}N HMBC experimental parameters for additional verification of the proposed imide bond in **1**, a doubling of the proton resonances appeared suggesting that the metabolite had begun to degrade. Purification by HPLC yielded two products, nygerone B (**2**)[3] and 2-methylsuccinate (**3**)[4] (Fig. 3) The fortuitous appearance of **2** as a hydrolysis product was key to confirming the

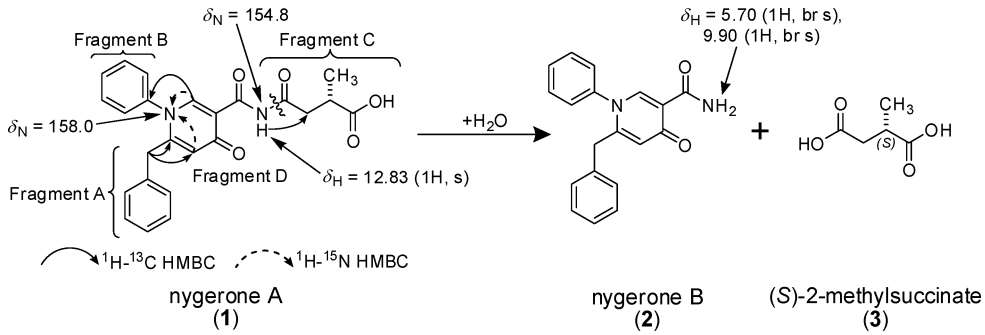

Fig. 3 Key long-range hetero-nuclear correlation (^{1}H-^{13}C HMBC: solid curved arrows and ^{1}H-^{15}N HMBC: broken curved arrows) and other NMR spectroscopic data used to assemble fragments A-D into **1**. The ^{1}H NMR data of **1** showed a single proton (δ_H 12.83) attached to the imide nitrogen (N-15), while hydrolysis of the N-15 to C-16 bond yielded **2** which displayed two hydrogens attached to the resulting amide nitrogen (δ_H 5.70 and 9.90). The other hydrolysis product, **3**, was identified as (*S*)-2-methylsuccinate based on comparisons of its ESIMS, ^{1}H NMR, and optical rotation data to authentic samples of the *R* and *S* isomers of **3**.

presence of the imide bond in **1** since the N-15 nitrogen atom now exhibited two attached amide protons at δ_H 5.70 (1H, br s) and 9.90 (1H, br s). Interestingly, a similar regio-specific imide hydrolysis process was previously observed as part of the degradation pathway for the quinolactacins from *Penicillium citrinum.*[5] Thus, we were able to rule-out an anhydride linkage between fragments C and D and confirm the presence of the imide linkage as illustrated for **1** (Fig. 1). With the planar structure of **1** determined, only the absolute configuration of the C-18 asymmetric center remained to be addressed. Polarimetry performed on the purified degradation product **3** gave an optical rotation of $[\alpha]_D^{21}$ −13.3 that was identical to an authentic sample of (*S*)-2-methylsuccinate. Therefore, the structure of the new γ-pyridone metabolite, nygerone A, produced by *A. niger via* chemical epigenetic induction was established as shown for **1**.

Metabolite **1** belongs to a growing class of unique fungal metabolites that are related to one another by virtue of their shared 4-oxo-1,4-dihydropyridine-3-carboxamide and 4-oxo-4H-pyran-3-carboxamide core structures. Notably, all of these metabolites are substituted at their respective C-6 positions, while C-2 and C-4 are consistently unsubstituted. The first γ-pyrone-containing members of this group of secondary metabolites, microsphaerones A and B, were reported in 2002 from the fermentation of a marine-sponge-derived *Microsphaeropsis* sp.[6] In 2004, the first γ-pyridone metabolite, aspernigrin A, was described from a marine-sponge-derived culture of *A. niger*; however, the structure was originally misassigned as containing an α-pyridone functionality.[7,8] The revised structure of aspernigrin A was proposed soon after by Ye and colleagues in 2005[9] based on a detailed investigation of NMR and X-ray crystallographic data obtained following the re-isolation of the same compound from an endophytic *Cladosporium herbarum.* Three γ-pyridone- and γ-pyrone-containing compounds, hemeic acids A-C,[10] were reported in 2005 from a marine-mussel-derived *Aspergillus* sp.; however, these metabolites are very structurally reminiscent of the microsphaerones.[6] It was not until 2007 that the γ-pyrone homolog of aspernigrin A called carbonarone A and its unique re-arrangement product, carbonarone B, were both isolated from a marine-sediment-derived *Aspergillus carbonarius.*[11] In addition to our disclosure of **1**, additional fungal-derived γ-pyridone- and γ-pyrone metabolites characterized as of 2008 include the berkeleyamides from an acid-lake-derived *Penicillium rubrum*[12] and pestalamides A-C isolated from a plant pathogenic strain of *Pestalotiopsis theae.*[13] Given the rapidly expanding structural diversity within this group of fungal metabolites (*vide supra*), considerable opportunity exists for the discovery of additional nygerone derivatives in the near future. One of the significant features of our approach to the isolation of **1** was the utilization of a chemical epigenetics methodology for inducing the production of this cryptic compound from our *A. niger* strain. Indeed, further examination of the fungus grown under different fermentation conditions demonstrated that **1** is produced exclusively upon treatment of the culture with the histone deacetylase inhibitor. In light of this result, it is clearly evident that chemical epigenetic techniques will be indispensable for finding additional nygerone derivatives and discovering new cryptic fungal metabolites that are encoded for by other transcriptionally recalcitrant biosynthetic pathways.

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Notes and references

1 R. H. Cichewicz, presented in part at the 2008 Annual Meeting of the Society for Industrial Microbiology, San Diego, August 2008.
2 R. B. Williams, J. C. Henrikson, A. R. Hoover, A. E. Lee and R. H. Cichewicz, *Organic & Biomolecular Chemistry*, 2008, **6**, 1895–1897.
3 Nygerone B (**2**): ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 3.66 (2H, s, H-7), 5.70 (1H, br s, N-H_a-15), 6.48 (1H, s, H-2), 6.80 (2H, m, H-9/13), 7.05 (2H, d, J = 7.6 Hz, H-2′/6′), 7.17 (2H, m, H-10/12), 7.18 (1H, m, H-11), 7.40 (2H, t, J = 7.6 Hz, H-2′/5′), 7.47 (1H, d, J = 7.6 Hz, H-4′), 9.90 (1H, br s, N-H_b-15); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C 39.2 (C-7), 118.1 (C-3), 121.8 (C-5), 126.9 (C-2′/6′), 127.5 (C-11), 128.7 (C-9/13), 128.7 (C-10/12), 129.7 (C-3′/5′), 130.0 (C-4′), 134.7(C-8), 140.4 (C-1′), 146.9 (C-2), 150.9 (C-6), 166.0 (C-14), 177.8 (C-4); HRESIMS m/z 305.1292 $[M + H]^+$ (calc. for $C_{19}H_{17}N_2O_2$ 305.1290).

4 (*S*)-2-methylsuccinate (**3**): $[\alpha]_D^{21}$ −13.3 (*c* 1×10^{-5}, MeOH); ^{1}H-NMR (400 MHz, CD_3OD) δ_H 1.21 (3H, d, $J = 7.0$ Hz), 2.39 (1H, dd, $J =$ 5.9 Hz, 16.9 Hz), 2.66 (1H, dd, $J = 8.6$ Hz, 16.9 Hz), 2.82 (1H, m).
5 B. Clark, R. J. Capon, E. Lacey, S. Tennant and J. H. Gill, *Organic & Biomolecular Chemistry*, 2006, **4**, 1512–1519.
6 C. Y. Wang, B. G. Wang, G. Brauers, H. S. Guan, P. Proksch and R. Ebel, *Journal of Natural Products*, 2002, **65**, 772–775.
7 J. Hiort, K. Maksimenka, M. Reichert, S. Perovic-Ottstadt, W. H. Lin, V. Wray, K. Steube, K. Schaumann, H. Weber, P. Proksch, R. Ebel, W. E. G. Muller and G. Bringmann, *Journal of Natural Products*, 2004, **67**, 1532–1543.
8 J. Hiort, K. Maksimenka, M. Reichert, S. Perovic-Ottstadt, W. H. Lin, V. Wray, K. Steube, K. Schaumann, H. Weber, P. Proksch, R. Ebel, W. E. G. Muller and G. Bringmann, *Journal of Natural Products*, 2005, **68**, 1821–1821.
9 Y. H. Ye, H. L. Zhu, Y. C. Song, J. Y. Liu and R. X. Tan, *Journal of Natural Products*, 2005, **68**, 1106–1108.
10 S. Tsukamoto, H. Hirota, M. Imachi, M. Fujimuro, H. Onuki, T. Ohta and H. Yokosawa, *Bioorganic & Medicinal Chemistry Letters*, 2005, **15**, 191–194.
11 Y. Zhang, T. Zhu, Y. Fang, H. Liu, Q. Gu and W. Zhu, *Journal of Antibiotics*, 2007, **60**, 53–157.
12 A. A. Stierle, D. B. Stierle and B. Patacini, *Journal of Natural Products*, 2008, **71**, 856–860.
13 G. Ding, L. Jiang, L. Guo, X. Chen, H. Zhang and Y. Che, *Journal of Natural Products*, 2008, **71**, 1861–1865.

