J|A|C|S COMMUNICATIONS

Published on Web 08/16/2010

Gene Expression Enabling Synthetic Diversification of Natural Products: Chemogenetic Generation of Pacidamycin Analogs

Abhijeet Deb Roy, Sabine Grüschow, Nickiwe Cairns, and Rebecca J. M. Goss*

School of Chemistry, University of East Anglia, Norwich NR4 7TJ, U.K.

Received July 8, 2010; E-mail: r.goss@uea.ac.uk

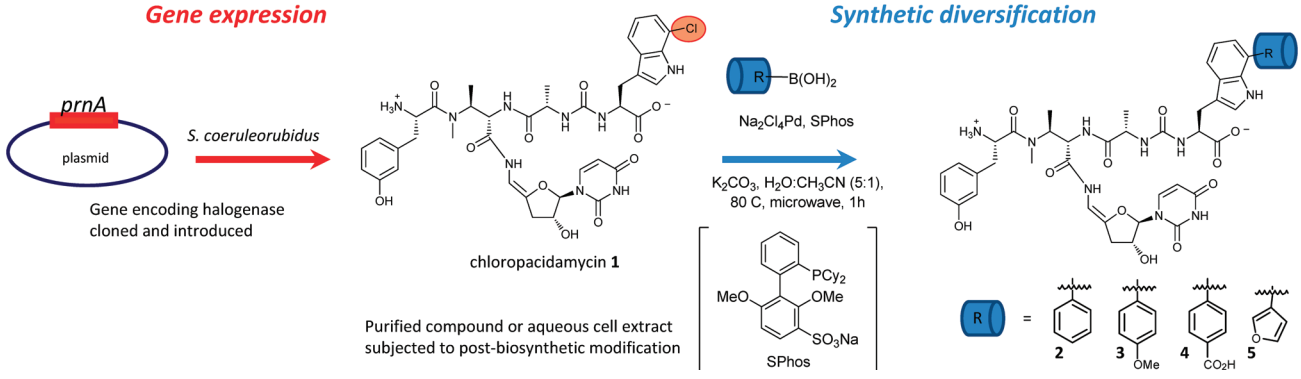
Abstract: Introduction of *prnA*, the halogenase gene from pyrrolnitrin biosynthesis, into *Streptomyces coeruleorubidus* resulted in efficient *in situ* chlorination of the uridyl peptide antibotic pacidamycin. The installed chlorine provided a selectably functionalizable handle enabling synthetic modification of the natural product using mild cross-coupling conditions in crude aqueous extracts of the culture broth.

Natural products represent a treasure trove of medicinally relevant compounds: over the past 3 decades over 70% of antimicrobials and over 60% of antitumor agents entering clinical trials have been based on natural products.[1] Generation of natural product analogs is an important area enabling improvement of activity and overcoming poor physicochemical properties. Conventional methods of analog generation include total synthesis, semisynthesis, and mutasynthesis.[2]

Here we describe a new paradigm in natural product analog generation, the introduction of a gene to act in concert with an existing biosynthetic pathway so as to install a chemical handle enabling selective functionalization of the natural product (Scheme 1). The biosynthetic introduction of a halogen into a compound affords the, until now unrealized, potential to enable selective postbiosynthetic derivatization. Over the past 5 years the structures of a series of halogenases have been reported.[3−6] Four examples of the heterologous expression of halogenases have been described so far. These reports involve the introduction of a halogenase into closely structurally and biosynthetically related systems and the generation of additional analogs of known halometabolites.[7] We report the first introduction of a halogenase to complement a genetically undefined and biosynthetically unrelated pathway giving access to a new series of halometabolites. The postbiosynthetic diversification of these new unnatural products is also communicated. As the test bed for these experiments, we have chosen the uridyl peptide antibiotic (UPA) pacidamycin, a compound with an unusual structure and with good activity against the Gram-negative pathogen *Pseudomonas aeruginosa*.[8] No natural halogenated UPA analogs exist. At the time of carrying out these experiments the pacidamycin producer, *Streptomyces coeruleorubidus* AB11383F-64, was genetically uncharacterized. Although little is known about the biosynthesis of these antibiotics, the enzymes involved in the assembly of pacidamycin have been shown to be highly flexible and able to incorporate a series of unnatural substrate analogues.[9,10]

To enable the realization of the concept two challenges needed to be addressed: first the introduction and expression of a gene that would produce an active protein capable of acting in concert with the natural biosynthetic machinery to introduce a halogen; second application of sufficiently mild and aqueous derivatization conditions to enable selective cross-coupling chemistry at the halogen without decomposition of the thermally unstable natural product. As we had previously demonstrated the striking promiscuity of the pacidamycin biosynthetic enzymes toward 7-halotryptophans,[9] we undertook to explore the introduction of *prnA*, the tryptophan 7-halogenase gene from pyrrolnitrin biosynthesis, to complement the pacidamycin biosynthetic genes.[3] The gene was cloned into the integrative plasmid pIJ10257[11] in which *prnA* was placed under the control of the constitutive promoter ermE*. The expression construct was introduced into the pacidamycin producer *S. coeruleorubidus* through conjugation from *Escherichia coli* to give strain RG-5059. The resulting strain produced chloropacidamycin **1** (Scheme 1) alongside nonhalogenated pacidamycins. The isolated yields of chloropacidamycin **1** were approximately 1 mg per liter culture. This titer is comparable to **1** obtained from precursor-directed biosynthesis.[9] Typically, the ratio of **1** to its parent pacidamycin was 1:5 but could go as high as 4:1 in favor of the

Scheme 1. Gene Expression Enabling Synthetic Diversification[a]



[a] The introduction of *prnA*, a gene encoding a halogenase, into the pacidamycin producer results in the generation of chlorinated pacidamycins. The chlorine may be used as a selectably functionalizable handle enabling further synthetic diversification.

chlorinated pacidamycin. The factors governing the observed variability are currently not fully understood.

We then turned our attention to the chemical functionalization of the halogen handle and the possibility of derivatizing the natural product as a component of a crude aqueous extract. We envisaged that the introduction of the chlorine to the pacidamycin skeleton would make it a suitable substrate for Pd-mediated Suzuki−Miyaura and related cross-coupling reactions. Pacidamycin is a particularly demanding, and therefore useful, model for synthetic derivatization; it contains a number of potentially reactive functional groups that render selective synthetic modifications difficult, it is thermally unstable,[8] and due to its highly polar nature it is insoluble within organic solvents. So far, few examples have been reported of the palladium-catalyzed cross-coupling reactions of hydrophilic aryl chlorides with aryl boronic acids under aqueous conditions. The few reported examples employ high temperatures and long reaction times, making these conditions incompatible with thermally unstable natural products.[12] Therefore, we sought to develop milder reaction conditions to enable the Suzuki−Miyaura coupling of chlorinated natural products.

Initially, we explored the cross-coupling of unprotected 7-chlorotryptophan as the model compound. With our previously developed conditions using tris(3-sulfonatophenyl)phosphine trisodium salt (TPPTS) as ligand[13] we were unable to effect the cross-coupling of this relatively unreactive species; however, by applying extended heating in a microwave up to 10% conversion could be observed. In order to improve the reaction we explored a range of water-soluble catalysts and ligands (Supporting Information (SI)) and found the sterically more demanding and electron-rich SPhos ligand, designed by Buchwald, to work best.[14] While direct application of the reported Buchwald conditions to 7-chlorotryptophan led to no detectable product, by investigating different palladium salts and adding a small amount of acetonitrile to solvate SPhos we were able to determine conditions that enabled rapid reaction with 7-chlorotryptophan (see SI).

Having optimized conditions that would facilitate the cross-coupling of the 7-chlorotryptophan, we investigated their application to the modification of chloropacidamycin **1**. Cross-coupling using boronic acid, Na_2Cl_4Pd-SPhos, and K_2CO_3 in water/acetonitrile under microwave heating at 80 °C for 1 h allowed the modification of not only purified chloropacidamycins but also crude extracts of **1**. The Suzuki−Miyaura cross-coupling was explored with four boronic acids. Reaction with phenyl boronic acid or 4-methoxybenzene boronic acid resulted in quantitative conversion of **1** to give **2** and **3**, respectively (Figure 1). Unsurprisingly, lower conversions were observed with the more electron-deficient 4-ethoxycarbonyl phenylboronic acid. During this reaction the ethyl ester was cleaved resulting in the corresponding carboxylic acid derivative **4** being formed as the major product. Remarkably, the cross-coupling was equally effective with the less reactive furan-2-boronic acid to give **5**. Reaction products were analyzed by LC-MS/MS. Three fragments proved to be diagnostic for the presence of additional substituents on the tryptophan residue (Figure 1).

Economical access to natural product analogs is an essential component of drug discovery. We have demonstrated that a gene can be expressed in complement to a genetically undefined biosynthetic pathway installing a handle that enables further selective synthetic diversification. The derivatization of the resulting natural product has been achieved through the application of exceptionally mild cross-coupling conditions. This has enabled the modification of the natural product within aqueous extracts of the fermentation broth, and in its semipurified and purified states. The applicability of this approach to a genetically undefined system,

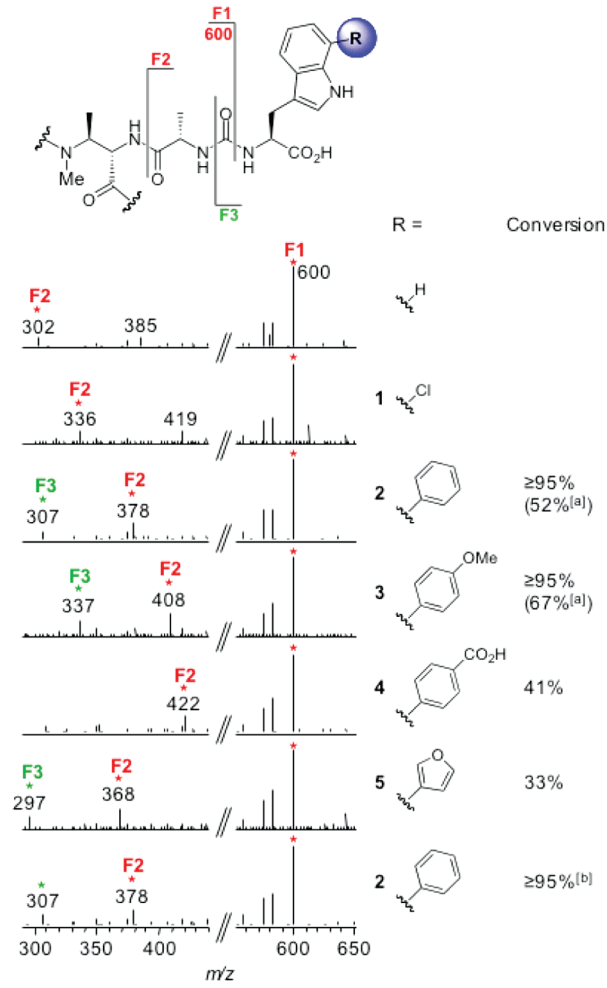


Figure 1. MS/MS analysis of pacidamycin analogs. Diagnostic fragment ions are shown. Percent conversion based on consumption of **1** is listed next to the corresponding partial spectra. [a] denotes isolated yield; [b] the reaction was carried out on **1** as a component of a crude aqueous extract.

and a highly functionalized and thermally unstable natural product implies great potential for this technology.

Acknowledgment. We are grateful to Prof. K.-H. van Pée (TU Dresden) and Prof. M. J. Bibb (John Innes Centre) for provision of plasmids pCIB7805 and pIJ10257, respectively. We thank Dr. L. M. Hill (JIC) and the National Mass Spectrometry Service (Swansea) for MS analysis. We are grateful to the Royal Society for an RS Dorothy Hodgkin Fellowship for R.J.M.G. and to The Leverhulme Trust for their support of this project F/00204/AO, F/00204/AF.

Supporting Information Available: Full experimental details and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461.
(2) For example, see: (a) Nising, C. F.; Brase, F. *Angew. Chem., Int. Ed.* **2008**, *47*, 9389. (b) Cottreau, K. M.; Spencer, C.; Wentzell, J. R.; Graham, C. L.; Borissow, C. N.; Jakeman, D. L.; McFarland, S. A. *Org. Lett.* **2010**, *12*, 1172. (c) Runguphan, W.; O'Connor, S. E. *Nat. Chem. Biol.* **2009**, *5*, 151.
(3) Dong, C.; Flecks, S.; Unversucht, S.; Haupt, C.; van Pée, K. H.; Naismith, J. H. *Science* **2005**, *309*, 2216.
(4) Blasiak, L. C.; Vaillancourt, F. H.; Walsh, C. T.; Drennan, C. L. *Nature* **2006**, *440*, 368.
(5) Dong, C. J.; Huang, F. L.; Deng, H.; Schaffrath, C.; Spencer, J. B.; O'Hagan, D.; Naismith, J. H. *Nature* **2004**, *427*, 561.

(6) Eustáquio, A. S.; Pojer, F.; Noel, J. P.; Moore, B. S. *Nat. Chem. Biol.* **2008**, *4*, 69.
(7) (a) Eustáquio, A. S.; Gust, B.; Luft, T.; Li, S. M.; Chater, K. F.; Heide, L. *Chem. Biol.* **2003**, *10*, 279. (b) Sánchez, C.; Zhu, L.; Braña, A. F.; Salas, A. P.; Rohr, J.; Méndez, C.; Salas, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 461. (c) Heide, L.; Westrich, L.; Anderle, C.; Gust, B.; Kammerer, B.; Piel, J. *ChemBioChem* **2008**, *9*, 1992. (d) Eustáquio, A. S.; O'Hagan, D.; Moore, B. S. *J. Nat. Prod.* **2010**, *73*, 378.
(8) Chen, R. H.; Buko, A. M.; Whittern, D. N.; McAlpine, J. B. *J. Antibiot.* **1989**, *42*, 512.
(9) Grüschow, S.; Rackham, E. J.; Elkins, B.; Newill, P. L. A.; Goss, R. J. M. *ChemBioChem* **2009**, *10*, 355.
(10) Ragab, A. E.; Grüschow, S.; Rackham, E. J.; Goss, R. J. M. *Org. Biomol. Chem.* **2010**, *8*, 3128.
(11) Hong, H. J.; Hutchings, M. I.; Hill, L. M.; Buttner, M. J. *J. Biol. Chem.* **2005**, *280*, 13055.
(12) Anderson, K. W.; Buchwald, S. L. *Angew. Chem., Int. Ed.* **2005**, *119*, 6329.
(13) Deb Roy, A.; Goss, R. J. M.; Wagner, G. K.; Winn, M. *Chem. Commun.* **2008**, 4831.
(14) Barder, T. E.; Walker, S. D.; Martinelli, J. R.; Buchwald, S. L. *J. Am. Chem. Soc.* **2005**, *127*, 4685.

JA1060406