

CHEMISTRY

A European Journal

A Journal of



Accepted Article

Title: Monomeric Dihydroanthraquinones: A Chemoenzymatic Approach and its (Bio)synthetic Implications for Bisanthraquinones

Authors: Nirmal Saha, Amit Mondal, Karina Witte, Shailesh Kumar Singh, Michael Müller, and Syed Masood Husain

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *Chem. Eur. J.* 10.1002/chem.201705998

Link to VoR: <http://dx.doi.org/10.1002/chem.201705998>

Supported by
ACES

WILEY-VCH

COMMUNICATION

Monomeric Dihydroanthraquinones: A Chemoenzymatic Approach and its (Bio)synthetic Implications for Bisanthraquinones

Nirmal Saha⁺,^[a] Amit Mondal⁺,^[a] Karina Witte,^[a,b] Shailesh Kumar Singh,^[a] Michael Müller,^[b] and Syed Masood Husain*^[a]

Abstract: Modified bisanthraquinones are complex dimeric natural products containing a cage-like structural motif. For their biosynthesis, monomeric dihydroanthraquinones have been proposed as key intermediates despite not being isolated from natural sources or synthesized as yet. Herein, isolation and characterization of dihydroemodin, as well as dihydrolunatin, synthesized via a biomimetic, chemoenzymatic approach using NADPH-dependent polyhydroxyanthracene reductase (PHAR) from *Cochliobolus lunatus* followed by Pb(OAc)₄ oxidation is reported. Subsequent dimerization via a hetero-Diels–Alder reaction of the dihydroemodin and dihydrolunatin resulted in (–)-flavoskyrin (68%) and (–)-lunaskyrin (62%), respectively. Pyridine treatment of (–)-flavoskyrin and (–)-lunaskyrin gave (–)-2,2'-*epi*-cytoskyrin A in 64% and 60% yield, respectively, through a cascade that involves two dimeric intermediates. Implications of the described synthesis for the biosynthesis of bisanthraquinones by a combination of enzymatic and spontaneous steps are discussed.

Emodin (1), one of the most widely distributed anthraquinones, is found in various species of plants^[1] and fungi.^[2] It plays a crucial role as a biosynthetic precursor for many fungal metabolites such as the ergochromes (*Penicillium oxalicum*),^[3] xanthenes (*Aspergillus versicolor*),^[4] monodictyphenone (*Aspergillus nidulans*),^[5] and bisanthraquinones (*Penicillium islandicum*,^[6] *Myrothecium verucaria*,^[7] and *Penicillium rugulosum*).^[6c,8] The astonishing molecular diversity is due to the versatile chemical properties of anthraquinones: reduction or oxidation and subsequent transformations may provide easy access to a variety of monomeric and dimeric metabolites. For example, chrysophanol (2), a precursor in monodictyphenone biosynthesis, is formed by deoxygenation of emodin (1).^[4,5,9] Detailed investigation of the deoxygenation process by some of us led to the discovery of dihydroanthracen-1(2*H*)-one 3 as a crucial biosynthetic intermediate (Figure 1).^[10] Compound 3 is formed by the reduction of tautomers of emodin hydroquinone by NADPH-dependent

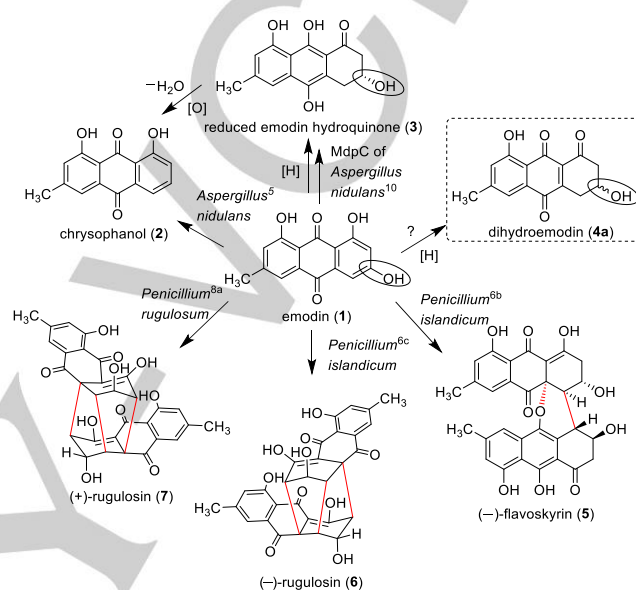


Figure 1. Monomeric biosynthetic intermediates and dimeric bisanthraquinones.

MdpC (from the monodictyphenone biosynthetic gene cluster of *A. nidulans*), which on elimination of water results in the formation of chrysophanol (2). The same transformation is catalyzed by analogous enzymes, AfIM from *Aspergillus parasiticus*^[11] and '17 β -hydroxysteroid dehydrogenase' (17 β -HSDcl) from *Cochliobolus lunatus*,^[12] which further led to the elucidation of deoxygenation steps in aflatoxin B₁^[11] and cladofulvin biosynthesis.^[13]

Another related biosynthetic intermediate, dihydroemodin (4a), has been proposed as being involved in the biosynthesis of (–)-flavoskyrin (5) and (–)-rugulosin (6), isolated from *P. islandicum*, and (+)-rugulosin (7), isolated from *P. rugulosum*.^[14,15] The proposal was based on a series of monomeric anthraquinones synthesized by Zahn and Koch almost 80 years ago, which tend to dimerize via a hetero-Diels–Alder reaction into flavoskyrin-type dimeric compounds.^[15b,16] This has been reflected in the biomimetic synthetic strategies to bisanthraquinones, evident from the cascade syntheses of (+)-rugulosin (7) and its analogues developed independently by the groups of Nicolaou^[17] and Snider, respectively.^[18] Interestingly, both approaches involved flavoskyrin-type dimeric intermediates which are formed by dimerization of monomeric anthrahydroquinones, followed by a synthetic cascade.^[17] However, the proposed monomeric intermediate, dihydroemodin (4a), and its naturally occurring dimer, flavoskyrin (5), could not be synthesized so far, owing to the presence of sensitive β -hydroxy ketone groups^[19] which may undergo elimination.

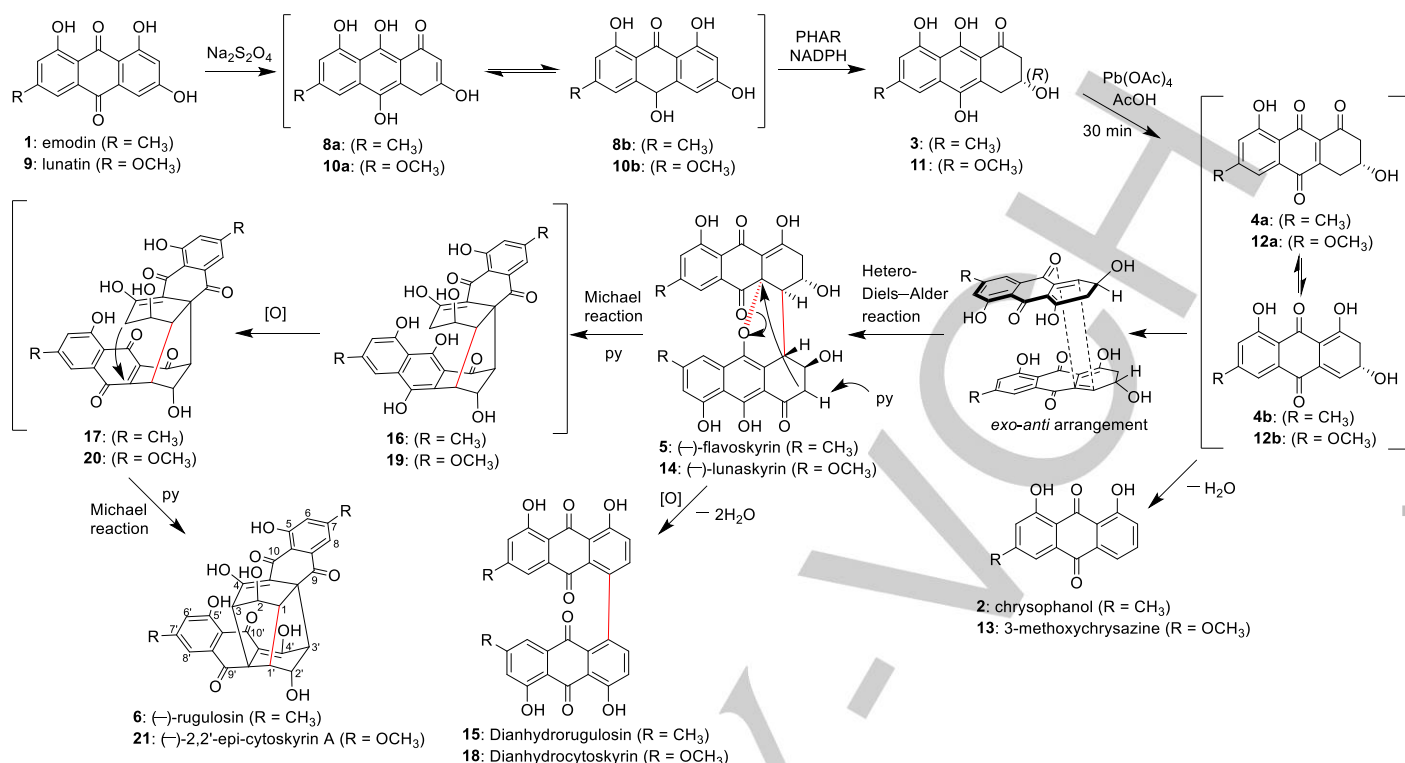
[a] N. Saha,* A. Mondal,* K. Witte, S. K. Singh, Dr. S. M. Husain
Molecular Synthesis and Drug Discovery Unit, Centre for Biomedical Research, Sanjay Gandhi Postgraduate Institute of Medical Sciences Campus, Raebareilly Road, Lucknow 226014 (India)
E-mail: smhusain@cbmr.res.in, smhusain.iitb@gmail.com

[b] K. Witte, Prof. Dr. M. Müller
Institut für Pharmazeutische Wissenschaften
Albert-Ludwigs-Universität Freiburg
Albertstrasse 25, 79104 Freiburg (Germany)

[*] These authors contributed equally to this work.

Supporting information for this article is given via a link at the end of the document. (Please delete this text if not appropriate)

COMMUNICATION



Scheme 1. Chemoenzymatic synthesis of (–)-rugulosin (**6**) and (–)-2,2'-epi-cytoskyrin A (**21**). Reduction of emodin (**1**) and lunatin (**9**) by His-tagged PHAR using NADPH (regenerated using glucose/glucose dehydrogenase) in the presence of Na₂S₂O₄. Oxidation of reduced anthrahydroquinones **3**, **11** by Pb(OAc)₄ in AcOH gave reduced quinone intermediates **4**, **12**, dimeric compounds **5** (68%), **14** (64%), and deoxygenation products **2** (8%), **13** (10%). Treatment with base converts **5**, **14** into modified bisanthraquinones **6** (64%), **21** (60%), and dianhydro compounds **15** (10%), **18** (12%), via intermediates **16**, **19** and oxidized **17**, **20**.

Therefore, the existence of **4a** and its role in the biosynthesis of flavoskyrin and rugulosin remained elusive. In order to resolve this long-pending issue, we focused on the synthesis of dihydroemodin (**4a**). We hypothesized that **4a** can be formed by oxidation of reduced emodin hydroquinone **3**. To test our hypothesis, we used 17β-HSDcl, synonymous with polyhydroxy-anthracene reductase (PHAR), from the filamentous fungus *Curvularia lunata* (teleomorph *C. lunatus*) to convert emodin (**1**) into **3**.^[12] Emodin (**1**) on incubation with Na₂S₂O₄ resulted in a tautomeric mixture of emodin hydroquinones **8a/8b**, which after reduction with PHAR in the presence of NADPH gave reduced emodin hydroquinone **3** in 75% yield (see Supporting Information) (Scheme 1). In this transformation, NADPH was regenerated using a glucose/glucose dehydrogenase system. To obtain **4a** from **3**, we first applied MnO₂ for oxidation, which mainly resulted in the formation of chrysophanol (**2**), with no trace of monomeric intermediate (**4a**). Alternatively, we used Pb(OAc)₄/AcOH, as employed by Seo et al.^[15a,b] and later by Snider and Gao,^[18] for the selective oxidation of anthrahydroquinones. Addition of a suspension of Pb(OAc)₄ in AcOH to an ice-cold suspension of **3** in AcOH and stirring for 20 minutes resulted in the formation of mainly two new products along with the expected chrysophanol (**2**), with no starting material remaining. Purification by preparative TLC provided a yellow compound in 30% yield (*R_f* = 0.43 MeOH/CHCl₃ 1:9), 8% chrysophanol (*R_f* = 0.90), and another (orange) compound (*R_f* = 0.46) which decomposed during isolation. Characterization of the yellow compound by NMR

spectroscopy, mass spectrometry, and CD revealed that it was identical to (–)-flavoskyrin (**5**, Scheme 1). Further reaction optimization and purification by chromatography with oxalic acid impregnated silica gel resulted in **5** in 68% yield (see Supporting Information).

In order to purify and identify the unknown product, chromatography with oxalic acid impregnated silica gel was used with benzene/acetone (9:1) as eluent, which ultimately led to the isolation of the orange product, monomeric dihydroemodin (**4a**) (see Supporting Information). The configuration of the chiral center in **4a** is assumed to be the same as that of **3**. In addition, dihydroemodin exists as a mixture of tautomers **4a** and **4b** in a ratio of 85:15 (¹H NMR, acetone-*d*₆) (Scheme 1). As the minor tautomer **4b** could not be isolated from the mixture, it was characterized by 1D-TOCSY NMR experiments (see Supporting Information).

Hence, the synthesis of monomeric dihydroemodin (**4a**) and its dimerized product (–)-flavoskyrin (**5**) was realized through a two-step chemoenzymatic approach. The results are consistent with the formation of (–)-flavoskyrin via the hetero-Diels–Alder reaction of two units of **4a/4b**, formed by oxidation of **3**. Biosynthesis of (–)-flavoskyrin (**5**) might involve similar steps, either catalyzed by enzymes or occurring spontaneously. It is important to note that the cycloaddition will take place according to an *exo-anti* arrangement as depicted in Scheme 1, to minimize the steric hindrance between the two hydroxyl groups. To ascertain the

COMMUNICATION

generality of these results and to obtain monomeric intermediates and flavoskyrin-type dimeric compounds of other unprotected anthraquinones, we envisaged the synthesis of 2,2'-*epi*-cytoskyrin A (**21**) starting from monomeric lunatin (**9**). Lunatin (**9**) has already been proposed as being involved in the biosynthesis of cytoskyrin A.^[12] Both lunatin and (+)-cytoskyrin A have been isolated from the fungus *C. lunata*^[20] and their biosynthesis could involve PHAR for the reduction of lunatin hydroquinones **10a/10b**.^[12] For this purpose, lunatin (**9**) was synthesized using 2,6-dichlorobenzoquinone and mixed vinyl ketene acetals via two consecutive Diels–Alder reactions in 32% yield (see Supporting Information). On reduction in the presence of Na₂S₂O₄ using the PHAR/NADPH system, **9** gave (*R*)-3,8,9,10-tetrahydroxy-6-methoxy-3,4-dihydroanthracen-1(2*H*)-one (**11**) in 64% yield. The configuration was assigned by comparing the CD spectra of **3** and **11**. In this transformation, 3-methoxychrysazine (**13**) was also isolated as a side product (10%), formed by oxidation and dehydration of **11** (see Supporting Information) (Scheme 1). Although **13** has yet to be isolated from fungi, its isolation has been reported from the plant *Xyris pilosa*.^[21] In analogy to emodin reduction, we assume that the tautomers **10a/10b** of lunatin hydroquinone are formed in situ and act as substrates for the enzymatic reduction to give **11**. Next, we subjected **11** to Pb(OAc)₄/AcOH oxidation. The reaction mixture was purified by chromatography with oxalic acid impregnated silica gel; benzene/acetone (9:1) as eluent gave the flavoskyrin-type dimeric compound (–)-lunaskyrin (**14**) and 3-methoxychrysazine (**13**) in 62% and 10% yield, respectively. Dimer **14** was characterized by NMR spectroscopy, mass spectrometry, and CD (see Supporting Information). To obtain dihydrolunatin (**12a**), the reaction mixture was purified directly after extraction, resulting in a tautomeric mixture of **12a** and **12b** in a ratio of 87:13 (¹H NMR, acetone-*d*₆). The existence of tautomer **12b** is supported by 1D-TOCSY NMR experiments (see Supporting Information). Neither dihydrolunatin (**12a/12b**) nor (–)-lunaskyrin (**14**) have been isolated from natural sources or synthesized so far. Nevertheless, their chemoenzymatic synthesis presented here renders this a general biomimetic strategy toward such dimeric compounds. Further, the ability of PHAR to catalyze the reduction of anthrahydroquinones supports the putative involvement of such enzymes in the biosynthesis of substituted bisanthraquinones.

Next, we turned our focus to transform (–)-flavoskyrin (**5**) and (–)-lunaskyrin (**14**) into (–)-rugulosin (**6**) and (–)-2,2'-*epi*-cytoskyrin A (**21**), respectively. For this purpose, **5** was dissolved in pyridine and kept for 19 hours at rt. This resulted in the formation of (–)-rugulosin (**6**) and (–)-dianhydrorugulosin (**15**) in a ratio of 90:10 (¹H NMR, DMSO-*d*₆) (Scheme 1). Purification by chromatography with oxalic acid impregnated silica gel and elution with benzene/acetone (9:1 to 8:2) gave **6** and **15** in 64% and 10% yield, respectively. (–)-Dianhydrorugulosin (**15**) can be formed by elimination of two water molecules from (–)-flavoskyrin (**5**), which has been proposed previously.^[15] Moreover, when the same reaction was performed under argon atmosphere, the yield of **6** was decreased considerably; instead, two new products were formed, as observed by TLC. On purification and characterization by NMR spectroscopy and mass spectrometry, the two compounds were assigned the structures **16** and **17** (Scheme 1).

These results are in contrast with the previously postulated biosynthesis of rugulosin.^[15c] Accordingly, we propose an alternative pathway for the conversion of **5** into **6**, in which a Michael-type addition in the presence of a base gives **16**, which subsequently oxidizes in air to form **17**. Another intramolecular Michael addition in **17** finally results in the formation of the third C–C bond to give (–)-rugulosin (**6**). Similarly, treatment of (–)-lunaskyrin (**14**) with pyridine under oxygen atmosphere resulted in the formation of (–)-dianhydrocytoskyrin (**18**) and (–)-2,2'-*epi*-cytoskyrin A (**21**), in 12% and 60% yield, respectively (Scheme 1). The spectroscopic data of **21** match those of (+)-2,2'-*epi*-cytoskyrin A isolated from the endophytic fungus *Diaporthe* sp.,^[22] except for the optical rotation, which was expected based on the (*R*)-configuration of **11** (see Supporting Information). The same reaction under argon atmosphere led to the isolation of **19**; however, all attempts to isolate intermediate **20** failed as it undergoes facile Michael addition to form **21**. The chemoenzymatic synthesis of (–)-lunaskyrin (**14**) and (–)-2,2'-*epi*-cytoskyrin A (**21**) described herein points toward their putative existence as natural products which are yet to be isolated (Scheme 1).

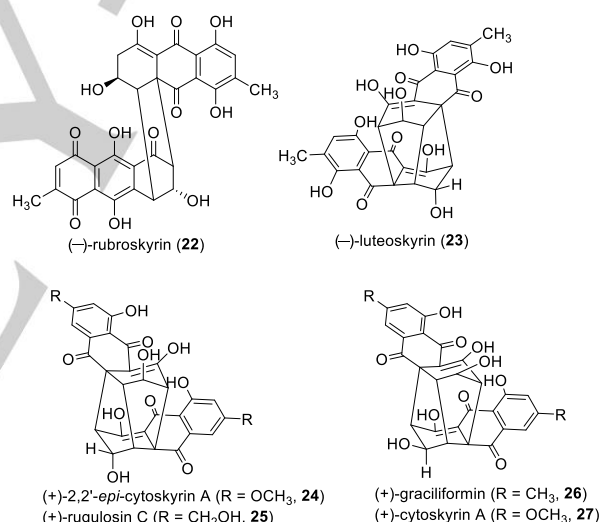


Figure 2. Other naturally occurring, modified bisanthraquinones.

Similar biosynthetic steps are expected for the formation of (+)-rugulosin C (**25**) isolated from *Penicillium radicum* using ω-hydroxyemodin as a starting point (Figure 2).^[24] The biosynthetic steps proposed here could be verified with the identification of the enzymes and their role in *P. islandicum*. The existence of (+)-graciliformin (**26**) isolated from *Cladonia graciliformis*^[25] and (+)-cytoskyrin A (**27**) isolated from *C. lunata*^[20] and the endophytic fungus CR200 (*Cytospora* sp.)^[26] require dimerization through an *exo-syn* arrangement, possibly by the involvement of enzyme(s) (Figure 2).

As no other flavoskyrin-type dimeric compound has been isolated from natural sources, it is conceivable that the dihydroanthraquinones undergo oxidative coupling by, for example, cytochrome

COMMUNICATION

P450 enzyme(s), without involving flavoskyrin. In addition, the role of spontaneity and aerial oxidation as a driving force for the formation of such complex molecules, which has been proposed in many biosynthetic pathways, cannot be ruled out.^[27] Hence, 'modified bisanthraquinones' might also be biosynthesized using nonenzymatic spontaneous reactions of biosynthetic intermediates, a notion that need further investigation.

Acknowledgements

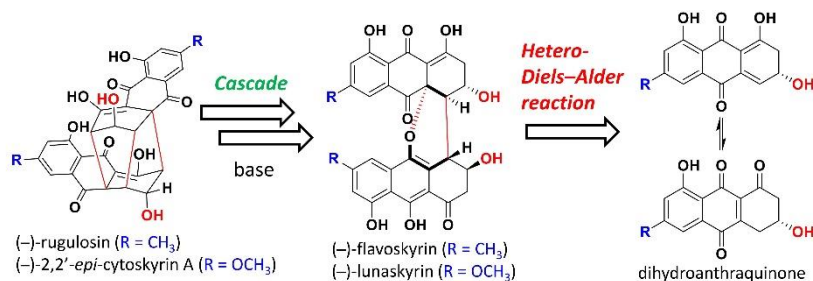
We are grateful to the Council of Scientific and Industrial Research, New Delhi (Project No. 02(0258)/16/EMR-II) and SERB-DST (YSS/2014/000792) for funding this research and fellowships to A.M. and N.S., respectively. We are thankful to Dr. Bikash Baishya and Ajay Verma for their expert help in NMR spectroscopy and Dr. Michael A. Schätzle and Dr. David Conradt for their helpful suggestions. We thank the Director, Centre of Biomedical Research for research facilities.

Keywords: Dihydroanthraquinones, modified bisanthraquinones, hetero-Diels–Alder reaction, cascade

- [1] I. Izhaki, *New Phytol.* **2002**, *155*, 205–217.
- [2] M. Fouillaud, M. Venkatachalam, E. Girard-Valenciennes, Y. Caro, L. Dufossé, *Mar. Drugs* **2016**, *14*, 1–64.
- [3] (a) B. Franck, F. Hüper, D. Gröger, D. Erge, *Chem. Ber.* **1968**, *101*, 1954–1969. (b) B. Franck, G. Bringmann, G. Flohr, *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 460–461.
- [4] (a) S. A. Ahmed, E. Bardshiri, T. J. Simpson, *J. Chem. Soc., Chem. Commun.* **1987**, 883–884. (b) S. Ahmed, E. Bardshiri, C. McIntyre, T. Simpson, *Aust. J. Chem.* **1992**, *45*, 249–274.
- [5] Y.-M. Chiang, E. Szewczyk, A. D. Davidson, R. Entwistle, N. P. Keller, C. C. C. Wang, B. R. Oakley, *Appl. Environ. Microbiol.* **2010**, *76*, 2067–2074.
- [6] (a) B. H. Howard, H. Raistrick, *Biochem. J.* **1954**, *56*, 56–59. (b) N. Takeda, S. Seo, Y. Ogihara, U. Sankawa, I. Iitaka, I. Kitagawa, S. Shibata, *Tetrahedron* **1973**, *29*, 3703–3719. (c) J. C. Bouhet, P. Pham Van Chuong, F. Toma, M. Kirszenbaum, P. Fromageot, *J. Agric. Food Chem.* **1976**, *24*, 964–972.
- [7] S. Nakamura, F. Nii, M. Shimizu, I. Watanabe, *Jpn. J. Microbiol.* **1971**, *15*, 113–120.
- [8] (a) J. Breen, J. C. Dacre, H. Raistrick, G. Smith, *Biochem. J.* **1955**, *60*, 618–626. (b) P. Sedmera, M. Podojil, J. Vokoun, V. Betina, P. Nemec, *Folia Microbiol. (Praha)* **1978**, *23*, 64–67.
- [9] (a) I. Kurobane, L. C. Vining, A. G. McInnes, J. A. Walter, J. L. C. Wright, *Tetrahedron Lett.* **1978**, 1379–1382. (b) T. J. Simpson, *ChemBioChem* **2012**, *13*, 1680–1688.
- [10] M. A. Schätzle, S. M. Husain, S. Ferlino, M. Müller, *J. Am. Chem. Soc.* **2012**, *134*, 14742–14745.
- [11] D. Conradt, M. A. Schätzle, J. Haas, C. A. Townsend, M. Müller, *J. Am. Chem. Soc.* **2015**, *137*, 10867–10869.
- [12] Cf. the suggested alternative annotation of 17 β -HSDcl as a polyhydroxy-anthracene reductase (PHAR): L. Fürtges, D. Conradt, M. A. Schätzle, S. K. Singh, N. Kraševac, T. L. Rižner, M. Müller, S. M. Husain, *ChemBioChem* **2017**, *18*, 77–80.
- [13] S. Griffiths, C. H. Mesarich, B. Saccomanno, A. Vaisberg, P. J. G. M. De Wit, R. Cox, J. Collemare, *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 6851–6856.
- [14] (a) S. Shibata, T. Murakami, I. Kitagawa, *Proc. Jpn. Acad.* **1956**, *32*, 356–360. (b) S. Shibata, T. Murakami, M. Takido, *Pharm. Bull.* **1956**, *4*, 303–308. (c) A. J. Birch, D. W. Cameron, R. W. Rickards, *J. Chem. Soc.* **1960**, 4395–4400. (d) J. A. Anderson, B.-K. Lin, H. J. Williams, A. I. Scott, *J. Am. Chem. Soc.* **1988**, *110*, 1623–1624.
- [15] (a) S. Seo, Y. Ogihara, U. Sankawa, S. Shibata, *Tetrahedron Lett.* **1972**, 735–736. (b) S. Seo, U. Sankawa, Y. Ogihara, Y. Iitaka, S. Shibata, *Tetrahedron* **1973**, *29*, 3721–3726. (c) D.-M. Yang, U. Sankawa, Y. Ebizuka, S. Shibata, *Tetrahedron* **1976**, *32*, 333–335. (d) U. Sankawa, In *The Biosynthesis of Mycotoxins*; P. S. Steyn, (Ed.); Academic Press: New York, **1980**; pp 357–394.
- [16] K. Zahn, H. Koch, *Ber. Dtsch. Chem. Ges. B* **1938**, *71*, 172–186.
- [17] (a) K. C. Nicolaou, Y. H. Lim, C. D. Papageorgiou, J. L. Piper, *Angew. Chem. Int. Ed.* **2005**, *44*, 7917–7921. (b) K. C. Nicolaou, Y. H. Lim, J. L. Piper, C. D. Papageorgiou, *J. Am. Chem. Soc.* **2007**, *129*, 4001–4013.
- [18] B. B. Snider, X. Gao, *J. Org. Chem.* **2005**, *70*, 6863–6869.
- [19] (a) K. C. Nicolaou, D. J. Edmonds, P. G. Bulger, *Angew. Chem. Int. Ed.* **2006**, *45*, 7134–7186. (b) K. C. Nicolaou, S. Rigol, *J. Antibiot.* **2017**; doi:10.1038/ja.2017.62.
- [20] R. Jadulco, G. Brauers, R. A. Edrada, R. Ebel, V. Wray, Sudarsono, P. Proksch, *J. Nat. Prod.* **2002**, *65*, 730–733.
- [21] B. Barros Cota, A. Braga de Oliveira, K. Guilherme Guimarães, M. Pimentel Mendonça, J. Dias de Souza Filho, F. Castro Braga, *Biochem. Syst. Ecol.* **2004**, *32*, 391–397.
- [22] A. Agusta, K. Ohashi, H. Shibuya, *Chem. Pharm. Bull.* **2006**, *54*, 579–582.
- [23] Y. Ueno, I. Ishikawa, *Appl. Microbiol.* **1969**, *18*, 406–409.
- [24] H. Yamazaki, N. Koyama, S. Omura, H. Tomada, *Org. Lett.* **2010**, *12*, 1572–1575.
- [25] H. Ejiri, U. Sankawa, S. Shibata, *Phytochemistry* **1975**, *14*, 277–279.
- [26] S. F. Brady, M. P. Singh, J. E. Janso, J. Clardy, *Org. Lett.* **2000**, *2*, 4047–4049.
- [27] E. Gravel, E. Poupon, *Eur. J. Org. Chem.* **2008**, 27–42.

COMMUNICATION

COMMUNICATION



Nirmal Saha, Amit Mondal, Karina Witte,
Shailesh Kumar Singh, Michael Müller,
Syed Masood Husain

Page No. – Page No.

**Monomeric Dihydroanthraquinones:
A Chemoenzymatic Approach and its
(Bio)synthetic Implications for
Bisanthraquinones**

Simple and elegant: (–)-flavoskyrin, (–)-lunaskyrin, (–)-rugulosin and (–)-2,2'-epi-cytoskyrin A were prepared in 2–3 steps from respective anthraquinones following a rationally designed chemoenzymatic synthesis.