NOTE

Protoilludane sesquiterpenoids as scaffold structures for new antimicrobials against *Mannheimia haemolytica*

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Research on the overall chemistry of basidiomycetes has led to the discovery of a wealth of secondary metabolites with rich variation of structures and novel carbon skeletons.¹ Some compounds have found medical applications as lead structures for the development of pharmaceutical products, starting material for agrochemicals or antibiotics.² Indeed, new antibiotics are continuously needed to counteract resistance of newly evolved pathogens and/or toxicity and allergic reactions of some of the current compounds.^{3,4}

Recently, two novel protoilludane sesquiterpenoids, named pasteurestins A and B (**1** and **2** in Figure 1), have been isolated and characterized.⁵ Both compounds were obtained by fermentation of the basidiomycete *Agrocybe aegerita* and were reported to show strong and selective activity against two strains of *Mannheimia haemolytica*, a pathogen causing bovine respiratory disease. Particularly, *M. haemolytica* is the principal bacterium isolated from respiratory disease in feedlot cattle and represents an important component in enzootic pneumonia in neonatal calves.⁶

This property made pasteurestins interesting as potential antibiotics in veterinary applications. Inspired from their biological activity, we were intrigued to evaluate suitable analogs as antimicrobials. The two pasteurestins are characterized by a highly strained hydrocyclobutane nucleus with five stereogenic centers. The complexity of the structures hampers the preparation of analogs by chemical synthesis. Indeed, Mulzer and colleagues⁷ have developed an elegant total synthesis of pasteurestins, consisting however of 20–22 linear steps with a 0.5–0.8% overall yield.

As part of our work on the search for unexplored resources, we have extensively investigated specific strains of basidiomycetes, such as species of *Armillaria*, *Clitocybe*, *Clavicornia* and *Echinodontium* (formerly classified as *Laurilia*). A large number of the characterized bioactive secondary metabolites belong to the group of sesquiterpenes. Typically, they present a differently substituted protoilludane skeleton to form a multitude of transformed and rearranged tricyclic

compounds.^{8–17} Among the isolated compounds, we selected those more structurally related to pasteurestins (compounds **3–14** in Figure 1) and available in sufficient amounts to perform antimicrobial tests. The antimicrobial activity was tested against *M. haemolytica* ATCC 14003, *Pasteurella multocida* ATCC 15743 and *Escherichia coli* ATCC 25922.

All the compounds showed no activity against *P. multocida* (for details see Supplementary Information). Similarly, Sulcatine A,¹⁰ Tsugicoline E,¹² Melledonale C,¹³ 3-epi illudol,⁸ illudalic acid,¹⁵ illudine M¹⁶ (**3**, **7**, **9**), **13**¹³ and **14**¹⁷ were not active against *M. haemolytica* (MIC 128–256 µg ml⁻¹). On the contrary, Sulcatine B,¹¹ Tsugicoline A,⁹ Tsugicoline C⁹ (**4–6**) and **8**¹⁸ showed significant activity against *M. haemolytica* (MIC 32 µg ml⁻¹). It is interesting to notice that the absence of a -CH₂OH group (**3** vs **4**) and a different fusion of rings (**14**) caused a complete loss of activity. The MIC against *E. coli* was 32 µg ml⁻¹ for all the compounds tested (**3–14**).

These findings are noteworthy, as Takeuchi *et al.*⁵ reported an MIC of 0.78 µg ml⁻¹ for pasteurestin B, whereas in the antimicrobial tests performed by Mulzer and colleagues⁷ *M. haemolytica* and *E. coli* remained unaffected by synthetic pasteurestins.

The promising results prompted us to select one of the most active compounds, Tsugicoline A (**5**) to delineate the essential structural requirements for activity against *M. haemolytica*. As the complexity of the molecule excluded total synthesis, we focused on its isolation from natural sources. The need for consistent amounts of the natural product to prepare differently substituted derivatives required the choice of a good producing strain and the optimization of the fungal growth conditions. Tsugicoline A was the major metabolite isolated from the fungus *Echinodontium tsugicola* (Henn. & Shirai) Imazeki supplied as CBS 248.51.⁹ A second strain of *E. tsugicola* was kindly supplied by Dr Yoshihito Shiono (Department of Bioresource Engineering, Yamagata University Japan, Yamagata, Japan).¹⁹ Unlike mitospore fungi, some basidiomycetes are not easy to cultivate on

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Figure 1 Structures of Pasteurestins A and B and related sesquiterpenes tested. ^aIn brackets are the reported MIC values against *M. haemolytica*.

substrates at high nutrient availability and high concentrations of simple monomeric nutrients (mono- or oligo-saccharides and aminoacids), as the development of mycelium in such artificial systems is negatively affected. Similarly, submerged cultivation (liquid culture and fermenter) and cultivation duration may influence the type and the production of the secondary metabolites. In our previous experiments, tsugicolines A–D were produced by *E. tsugicola* in Erlenmeyer flasks containing malt extract broth.⁹ The optimization of the amount of tsugicolines A produced by the fungus was obtained by screening several substrates containing different sugar/peptide ratio and we found the best substrate to be a carbohydrate-rich broth enriched till 2% glucose. Introducing a pre-culture step at mild osmotic stress led to faster production of secondary metabolites, compared with previous experiments (see Supplementary Information). Although both strains of *E. tsugicola* produced tsugicolines, differences in amount of production between them were observed. The crude extract obtained from the Japanese strain (750 mg l⁻¹) was greater than that of CBS strain (530 mg l⁻¹), but tsugicoline A (106 mg l⁻¹, 20% crude extract) was more abundant in CBS strain than in the Japanese one, which produced only 2.3% (17.5 mg l⁻¹). The compound mainly occurred in the liquid medium as the mycelium, dried and extracted separately, contained only traces

of tsugicolines. *E. tsugicola* CBS strain produced tsugicolines in liquid more than in agar cultures of the same medium (72 mg l⁻¹) where tsugicoline A represented only 7% of the crude extract. Solid state fermentation was not tried.

With the natural compound in hands, we planned to introduce modifications on the reactive groups of the molecule to perform initial structure-activity relationship studies.

We first tried to obtain a derivative with a carboxylic group on C-1, as in the pasteurestins, but several attempts to oxidize tsugicoline A (NaOCl -supported-TEMPO, NCS -supported TEMPO, MnO₂) were unsuccessful and gave inseparable mixtures of compounds. The introduction of the formyl group was accomplished only by treatment of **5** with TEA in MeOH to give compound **16**, most likely formed by the intermediate formation of an enolate, followed by water elimination⁹ (Figure 2). Further oxidation or catalytic reduction²⁰ of aldehyde **16** gave rise to complex mixtures of reaction products, thus confirming the sensitivity of this class of compounds to different reaction conditions.

Compound **17** was obtained by reduction of the carbonyl group with NaBH₄,¹⁷ whereas treatment of **5** with Ac₂O and pyridine at 0 °C afforded the corresponding triacetoxysterivative **18**.

Finally, reaction of **5** with different hydroxylamines gave the corresponding oximes **19a-c**. Compounds **16** and **17** showed good

Figure 2 Synthesis of Tsugicoline A derivatives. Reagents and conditions: (a) TEA, methanol, 4 h, room temperature, 43%; (b) NaBH₄, methanol, 2 h, room temperature, 79%; (c) acetic anhydride, dry pyridine, 0 °C, 6 h, 41%; (d) RONH₂.HCl, NaH₂PO₄, MeOH, room temperature, overnight.

activity (MIC 32 µg ml⁻¹), whereas compounds **18** and **19a-c** inhibited the growth of *M. haemolytica* with an MIC between 64 and 128 µg ml⁻¹.

These results allowed us to delineate some preliminary structure-activity relationship. First, they highlight the importance of the polar functions on C-1 and C-6. In fact, the introduction of the formyl group on C-1 retains the antimicrobial activity (**16** vs **5**), even if the OH on C-3 is removed, whereas acetylation of the three hydroxyl groups causes a decrease of the activity (**18** vs **5**). The keto group on C-5 seems to have a key role. In fact the natural compound 3-epi illudol (**10**), whose difference from tsugicoline is the absence of this group, shows lower activity. The activity is maintained if the carbonyl group is substituted with another polar group such as the hydroxyl group (**17**) or an unsubstituted oxime (**19a**). On the contrary, the introduction of bulky or lipophilic groups such as a methyl (**19b**) or a benzyl (**19c**) causes a decrease of activity.

In conclusion, Tsugicoline A, a sesquiterpenoid with protoilludane skeleton, was identified as a potential lead structure for the treatment of *M. haemolytica* and *E. coli* infections. Optimization of Tsugicoline A extraction from the fungus *E. tsugicola* allowed us to synthesize a series of derivatives and to develop preliminary structure-activity relationships studies to delineate the essential structural requirements for antimicrobial activity against *M. haemolytica*.

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- Schöffler, A. & Anke, T. Secondary Metabolites of Basidiomycetes. in *The Mycota* (eds Anke, T. & Weber, D.) XV 209–231 (Springer, Berlin, Heidelberg, New York, 2009).
- Aly, A. H., Debbab, A. & Proksch, P. Fifty years of drug discovery from fungi. *Fungal Divers.* **50**, 3–19 (2011).
- Pulcini, C. et al. Forgotten Antibiotics: An Inventory in Europe, the United States, Canada, and Australia. *Clin. Infect. Dis.* **54**, 268–274 (2012).
- Van der Poll, T. & Opal, S. M. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet.* **374**, 1543–1556 (2009).

- Takeuchi, T., Iinuma, H., Momose, I. & Matsui, S. (Jpn Kokai Tokkyo Koho). Antibiotic pasteurestin A and B and their manufacture with *Agroclybe cylindracea*. JP 2002212137 (2002).
- Rice, J. A., Carrasco-Medina, L., Hodgins, D. C. & Shewen, P. E. *Mannheimia haemolytica* and bovine respiratory disease. *Anim. Health Res. Rev.* **8**, 117–128 (2007).
- Kogl, M., Brecker, L., Warrass, R. & Mulzer, J. Novel protoilludane lead structure for veterinary antibiotics: total synthesis of pasteurestins A and B and assignment of their configurations. *Eur. J. Org. Chem.* **16**, 2714–2730 (2008).
- Arnone, A., Cardillo, R., Di Modugno, V. & Nasini, G. Secondary mould metabolites. Part 29. Isolation and structure elucidation of Candicansol, 3-epi-illudol and 1-O-Acetyl-3-epi-illudol, novel sesquiterpenoids from *Clitocybe candicans*, and absolute configuration of 3-epi-illudol. *J. Chem. Soc. Perkin Trans. 1*, 1995–2000 (1989).
- Arnone, A., Brambilla, U., Nasini, G. & Vajna de Pava, O. Isolation and structure elucidation of tsugicolines A-D, novel protoilludane sesquiterpenes from *Laurilia tsugicola*. *Tetrahedron* **51**, 13357–13364 (1995).
- Arnone, A., Nasini, G., Assante, G., Roeljmans, H. J. & Van Eijk, G. W. Secondary mould metabolites. Part XVIII. Sulcatine, a norsesquiterpene from the fungus *Laurilia sulcata*. *Phytochemistry* **26**, 1739–1742 (1987).
- Arnone, A., Nasini, G., Assante, G. & Van Eijk, G. W. Secondary mould metabolites. 35. Three sesquiterpenes produced by the fungus *Laurilia sulcata*. *Phytochemistry* **31**, 2047–2050 (1992).
- Arnone, A., De Gregorio, C., Meille, S. V., Nasini, G. & Sidoti, G. Tsugicoline E, a new polyoxygenated protoilludane sesquiterpene from the fungus *Laurilia tsugicola*. *J. Nat. Prod.* **62**, 51–53 (1999).
- Arnone, A., Cardillo, R. & Nasini, G. Secondary mold metabolites. Part 19. Structure elucidation and absolute configuration of melledonals B and C, novel antibacterial sesquiterpenoids from *Armillaria mellea*. X-ray molecular structure of melledonal C. *J. Chem. Soc. Perkin Trans. 1* **3**, 503–510 (1988).
- Arnone, A., Candiani, G., Nasini, G. & Sinisni, R. Isolation and structure elucidation of new sesquiterpenes of protoilludane origin from the fungus *Clavicornia divaricata*. *Tetrahedron* **59**, 5033–5038 (2003).
- Nair, M. S., Takeshita, H., McMorris, T. C. & Anchel, M. Metabolites of *Clitocybe illudens*. IV. Illudalic acid, a sesquiterpenoid, and illudinine, a sesquiterpenoid alkaloid. *J. Org. Chem.* **34**, 240–243 (1969).
- Anchel, M., Herve, A. & Robbins, W. J. Antibiotic substances from basidiomycetes: VII. *Clitocybe illudens*. *Proc. Natl Acad. Sci. USA* **36**, 300–305 (1950).
- Arnone, A., De Gregorio, C., Nasini, G. & Vajna de Pava, O. Secondary mould metabolites: part 53. Transformation of the protoilludane sesquiterpene tsugicoline A into a sterpurane derivative and its microbiological reduction. *J. Chem. Soc. Perkin Trans. 1*, 1523–1525 (1997).
- Arnone, A., De Gregorio, C., Mele, A., Nasini, G. & Vajna de Pava, O. Secondary mould metabolites. Part 58. Modifications in basic conditions and Michael additions of the protoilludane sesquiterpene tsugicoline A; some implications for the biogenesis of the sesquiterpenoids produced by Basidiomycetes. *J. Chem. Soc. Perkin Trans. 1*, 745–751 (2000).
- Shiono, Y., Suzuki, S., Murayama, T., Ikeda, M. & Abe, Y. Protoilludane sesquiterpenoids, echinocidins C and D produced by a decay causing fungal strain *Echinodontium tsugicola*. *Z. Naturforsch.* **60**, 449–452 (2005).
- Dallavalle, S., Merlini, L., Beretta, G. L., Tinelli, S. & Zunino, F. Synthesis and cytotoxic activity of substituted Luotonin A derivatives. *Bioorg. Med. Chem. Lett.* **14**, 5757–5761 (2004).

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