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Generation by mutasynthesis of potential neuroprotectant derivatives of the bipyridyl collismycin A

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ABSTRACT

Collismycin A is a member of the 2,2′-bipyridyl family of natural products and structurally belongs to the hybrid polyketides–nonribosomal peptides. A gene coding for a lysine 2-aminotransferase of *Streptomyces* sp. CS40 (collismycin A producer) was inactivated by gene replacement. The mutant was unable of synthesizing collismycin A but it recovered this capability when picolinic acid was added to the culture medium. By feeding different picolinic acid analogs to this mutant, two new collismycin A derivatives were obtained with a methyl group at the 4 and 6 position of the first pyridine ring of collismycin A, respectively. The two compounds showed effective neuroprotective action against an oxidative stress inducer in a zebra fish model, one of them showing higher neuroprotectant activity than that of collismycin A and that of the control lipoic acid.

The group of the 2,2′-bipyridyl-containing natural products is constituted by compounds sharing a two pyridine ring system which is further modified with some tailoring modifications. Members of this family include SF2738A-F,[1] pyrisulfoxins,[2] caerulomycins[3] and collismycins.[4] Different biological activities have been reported to be associated with compounds of this family including antibacterial, antifungal and cytotoxic activities[3–5] and collismycin A also to be an inhibitor of dexamethasone-glucocorticoid receptor binding and therefore potential as anti-inflammatory agent.[4] Biosynthetic studies with labeled precursors suggested that picolinic acid (PA) was a biosynthesis precursor which is derived from lysine.[6] The gene clusters for the biosynthesis of collismycin A from *Streptomyces* sp. CS40 and for the biosynthesis of caerulomycin from *Actinoalloteichus cyanogriseus* have been cloned and characterized in the last few years.[7] Sequence analysis and insertional inactivation experiments have shown that a hybrid polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) system is involved in the biosynthesis of these compounds.[7]

Collismycin A **1** (Fig. 1) contains the characteristic bipyridine ring system of the family and three substituents in the second pyridine ring: methoxy, methylthio and oxime groups. In the collismycin A cluster several genes were identified that could be involved in the biosynthesis of PA[7]: *clmL* possibly coding for a lysine 2-aminotransferase (LAT2) and the adjacent *clmS* that would code for a sarcosine oxidase-like in PA biosynthesis. The availability of PA analogues prompted us to consider the possibility of generating new collismycin A derivatives using a mutasynthesis approach. As a first step we verified the involvement of *clmL* and *clmS* in PA biosynthesis. The assumption was that *clmL* and *clmS* were responsible for the formation of PA from lysine: ClmL catalysing the transamination of lysine into 1-piperidine-2-carboxylic acid and ClmS oxidising this compound into PA (Fig. 2). To verify this hypothesis, we expressed in *Streptomyces albus* a 4.9 kb BamHI fragment under the control of the constitutive erythromycin resistance promoter *erm*E∗p and we looked for the formation of PA. This fragment contains both *clmL* and *clmS*, and a third gene *clmP* upstream of *clmL*, being transcribed in opposite direction and that would code for an acyl carrier protein of a type I polyketide synthase, with no role in PA biosynthesis. The resulting recombinant strain was grown and after 7 days of incubation at 30 °C, the cells

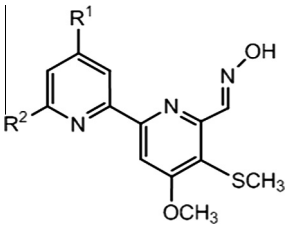

Figure 1. Chemical structure of collismycin A **1** ($R^1 = R^2 = H$) and the two new derivatives: collismycin M4 **2** ($R^1 = CH_3$; $R^2 = H$) and collismycin M6 **3** ($R^1 = H$; $R^2 = CH_3$).

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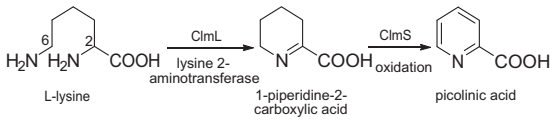

Figure 2. Proposed early steps in collismycin A biosynthesis from lysine to picolinic acid.

were broken by ultrasounds and extracted with perchloric acid (see Supplementary data). HPLC–MS analyses showed the presence of PA as compared with a commercial sample used as standard (Fig. 3), the levels being 28 times higher (Fig. 3) than those detected in cultures of *S. albus* only harbouring the vector (Fig. 3). These experiments confirm that these two genes, *clmL* and *clmS*, are involved in PA biosynthesis.

To generate new collismycin A derivatives by mutasynthesis we firstly generated a collismycin A nonproducing mutant by deleting *clmL* LAT2 gene thus producing an early block in the biosynthesis of collismycin A but still maintaining active the rest of the biosynthetic machinery. By gene replacement through the insertion of an apramycin resistance cassette (*aac(3)IV* gene) within the LAT2 gene, a mutant (CLM-L mutant) was generated that did not produce **1** (Fig. 4A) but it recovered this capability when 1 mM PA was added to the culture medium (Fig. 4B), indicating that all enzymatic machinery for the biosynthesis of **1** (with the exception of the LAT2 enzyme) was functional.

Then, we independently incubated the CLM-L mutant with 100 μg/ml of fourteen different PA analogues, all of them containing a free carboxylic acid group to establish the link to the second pyridine ring (Fig. 5). In this way we would expect new derivatives being formed in which the lysine-derived pyridine ring could be substituted by some of these analogues if there would be some kind of flexibility for the biosynthesis enzymes. UPLC and MS analyses of cultures of the different experiments showed that two of the analogues tested (4-methyl-PA 4 and 6-methyl-PA 5) were incorporated into new collismycin A derivatives (Fig. 1). These two compounds had a similar absorption spectrum to that of collismycin A and, as expected, 14 mass units more than **1**. The position of the methyl group at the 4′ and 6′ positions of the first ring of collismycin A was verified by NMR and MS. Moreover, the *E*-configuration of the oxime in both analogues **2** and **3** as well as in the parent collismycin A **1** was established based on a weak NOE observed between the iminic proton and the N–OH group. The remaining twelve analogues (compounds **6**–**17** in Fig. 5) tested were not apparently incorporated into **1** and they did not affect the normal growth of the mutant, with the exception of the four benzoic acid derivatives (compounds **10**–**13** in Fig. 5) that had to be used at lower concentrations (10 μg/ml) because toxicity at higher concentrations. The two new collismycin analogues, (*E*)-4-methoxy-4′-methyl-5-(methylthio)-[2,2′-bipyridine]-6-carboxaldehyde oxime **2** and (*E*)-4-methoxy-6′-methyl-5-(methylthio)-[2,2′-bipyridine]-6-carboxaldehyde oxime **3** were named collismycin M4 and collismycin M6, respectively. The yields of these compounds were in a range of 4.9–5.4 mg/l and 0.7–1.2 mg/l for compounds **2** and **3**, respectively. Yields for collismycin A were between 7.8 and 11.3 mg/l.

The two new derivatives were also tested as potential neuroprotectant agents in a zebrafish model.[8] Apoptosis was induced in neu-

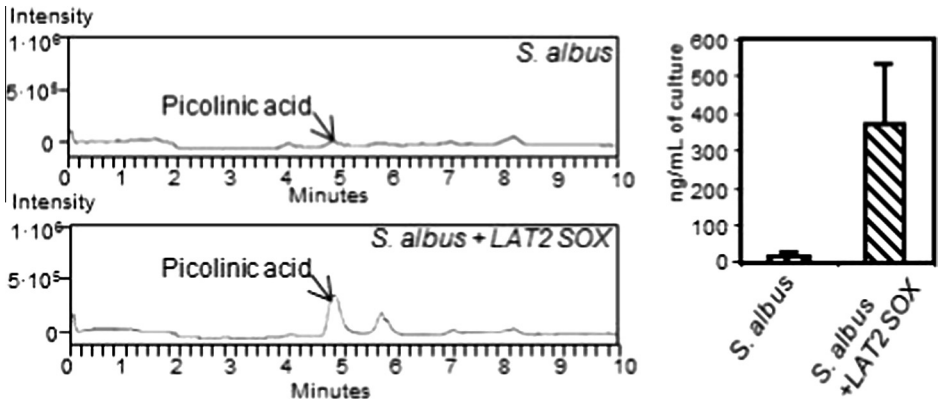

Figure 3. PA acid detection in *S. albus* +LAT2SOX and in *S. albus* (control; only containing vector pEM4T) and its quantitative estimation. All samples were run in triplicate and error bars represent confidence level of 95%.

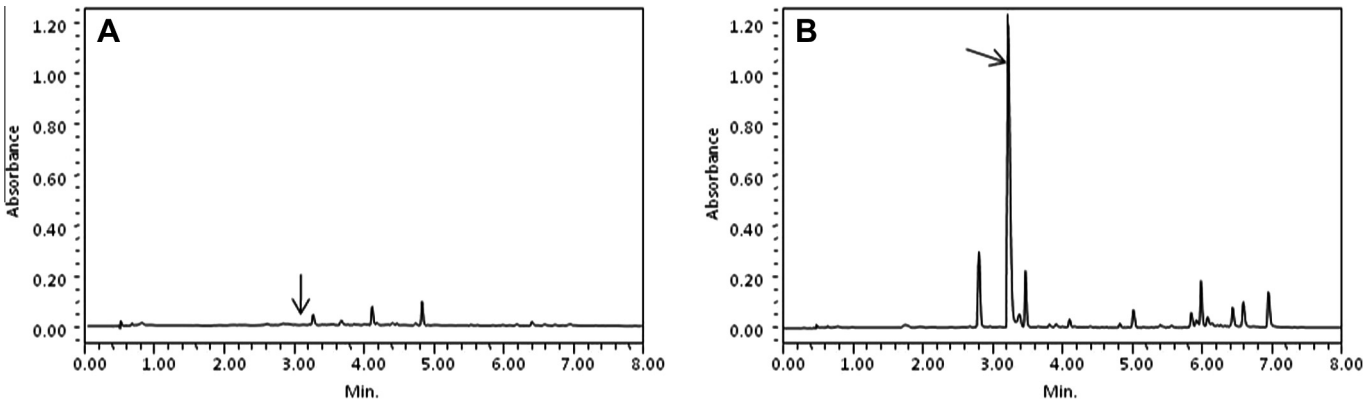

Figure 4. UPLC analyses of ethyl acetate extracts of cultures of *Streptomyces* sp. CLM-L mutant grown in the absence (A) or in the presence (B) of 1 mM PA. The arrow indicates the mobility of collismycin A.

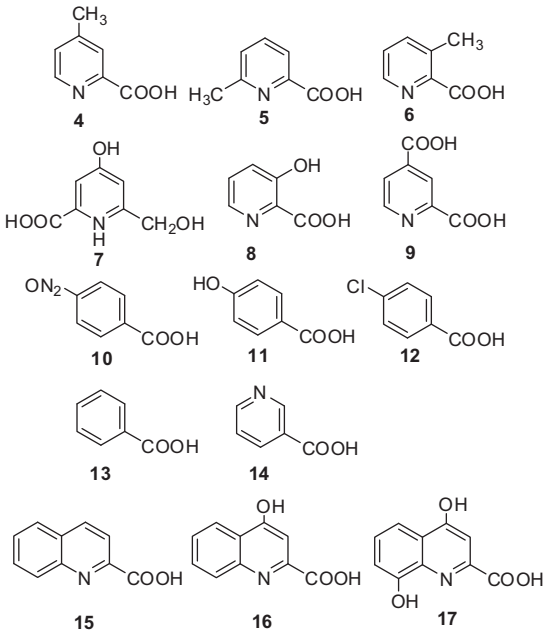

Figure 5. PA analogues tested as precursors for collismycin A biosynthesis in mutasynthesis experiments.

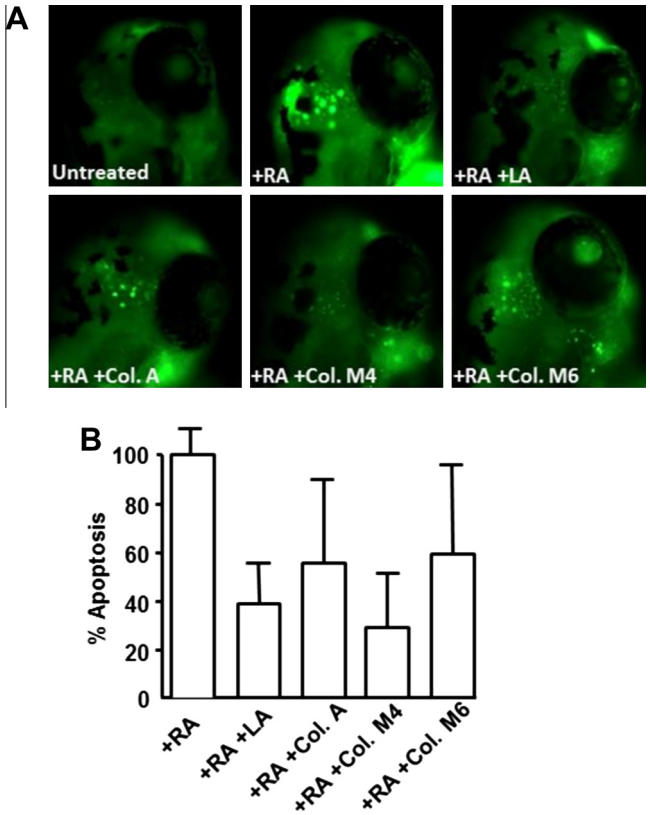

Figure 6. Assays of neuroprotection activity. (A) Induction of apoptosis as visualized by fluorescence microscopy. Induction was carried out by adding 10 μM RA to the zebra fish larvae and protection was done by adding 1 μM LA, collismycin A **1**, collismycin M4 **2** or collismycin M6 **3**. (B) Quantification of apoptosis induction. All analogs exhibited significant protection (P <0.05). Error bars represent confidence level of 95%.

ral cells using 3-dpf (days post fertilization) zebrafish larvae by incubation with 10 μM all-trans retinoic acid (RA) for 24 h in the presence or absence of 1 μM collismycin A, collismycin M4 or collismycin M6. Lipoic acid (thioctic acid) 1 μM was used as neuroprotectant positive control. Then, apoptotic cells were stained with 2 μg/ml acridinium chloride hemi-[zinc chloride] in fish water for 60 min and they were observed by fluorescence microscopy.[8] Apoptosis was quantified in 10 larvae per experiment and data submitted to statistical analysis (see Supplementary data). Control zebrafish larvae, with no RA treatment, exhibited few or no apoptotic cells (Fig. 6A), while RA-treated zebrafish exhibited a significant increase in apoptosis in the brain region (Fig. 6B). As a positive control, lipoic acid (LA) was used and pretreatment of the larvae with this compound reduced apoptosis in 61% (Fig. 6B). Pretreatment of the larvae with collismycin A reduced the appearance of apoptotic cells in 44% (Fig. 6B). The two new derivatives clearly differ in neuroprotectant activity: the 6-methyl derivative **3** showed similar neuroprotectant activity to that of collismycin A **1** (40% reduction in apoptosis; Fig. 6B) while the 4-methyl-derivative **2** showed higher level of protection (70% reduction in apoptosis; Fig. 6B).

In summary, by feeding different PA analogs to cultures of a mutant early blocked in the biosynthesis of PA in collismycin A biosynthesis, two new collismycin A derivatives, collismycin M4 **2** and collismycin M6 **3**, were generated showing neuroprotective activity. One of them showed higher activity than that of the parental collismycin A and of the positive control lipoic acid. These compounds might constitute leader compounds for further modifications in order to generate potential neuroprotective compounds.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures, NMR data for compounds **2** and **3**, copies of ^{1}H and ^{13}C NMR spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.08.017.

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