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Metabolic engineering of the heterologous production of clorobiocin derivatives and elloramycin in *Streptomyces coelicolor* M512

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Abstract

The aminocoumarin antibiotic clorobiocin is a potent inhibitor of bacterial gyrase. Two new analogs of clorobiocin could be obtained by deletion of a methyltransferase gene, involved in deoxysugar biosynthesis, from the biosynthetic gene cluster of clorobiocin, followed by expression of the modified cluster in the heterologous host *Streptomyces coelicolor* M512. However, only low amounts of the desired glycosides were formed, and aminocoumarins accumulated predominantly in form of aglyca. In the present study, we clarified the limiting steps for aminocoumarin glycoside formation, and devised strategies to improve glycosylation efficiency. Heterologous expression of a partial elloramycin biosynthetic gene cluster indicated that the rate of dTDP-L-rhamnose synthesis, rather than the rate of glycosyl transfer, was limiting for glycoside formation in this strain. Introduction of plasmid pRHAM which contains four genes from the oleandomycin biosynthetic gene cluster, directing the synthesis of dTDP-rhamnose, led to a 26-fold increase of the production of glycosylated aminocoumarins. Expression of the 4-ketoreductase gene *oleU* alone resulted in an 8-fold increase. Structural investigation of the resulting deoxysugars confirmed that both the endogeneous and the heterologous pathway involve a 3,5-epimerization of the deoxysugar, a hypothesis which had recently been questioned.

Keywords: Metabolic engineering; Aminocoumarin; Antibiotic; Deoxysugar; Biosynthesis; Streptomyces

1. Introduction

Combinatorial biosynthesis and metabolic engineering have become successful new tools for the development of new antibiotics, and may provide important strategies in the fight against antibiotic-resistant pathogens (Cane et al., 1998; Floss, 2006; Walsh, 2002). One principal limitation of this methodology, however, lies in the substrate specificity of some biosynthetic enzymes. If an early step in a pathway is modified in the producing organism by genetic methods, the resulting, structurally modified intermediates may not be accepted by downstream enzymes of the pathway with sufficient catalytic efficiency, resulting in a substantial reduction of the yields of the final product. We recently encountered such a problem in the metabolic engineering of aminocoumarin antibiotic biosynthesis. The aminocoumarins have previously provided an extraordinarily successful example for the generation of new bioactive compounds by combinatorial biosynthesis and metabolic engineering, often showing a considerable tolerance of their biosynthetic enzymes for structurally modified intermediates (Li and Heide, 2004, 2005).

However, a recent attempt to modify the pathway to the deoxysugar moiety of the aminocoumarins was less successful in terms of yields (Freitag et al., 2006). When we inactivated the methyltransferase gene *cloU* (Fig. 1) in the clorobiocin cluster and expressed the modified cluster in the heterologous host *Streptomyces coelicolor* M512, the resulting mutant accumulated predominantly the

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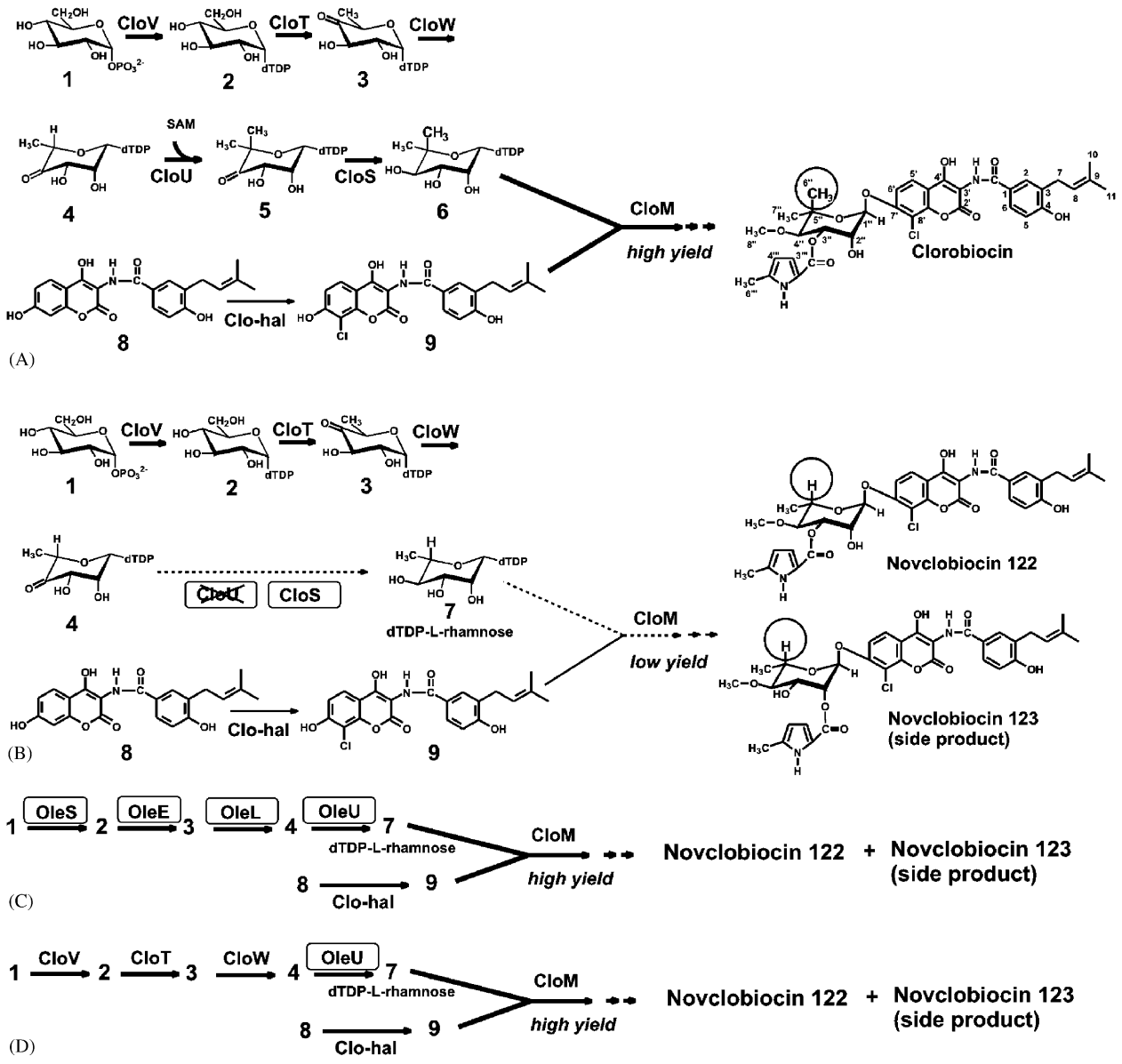

Fig. 1. Biosynthesis of aminocoumarin antibiotics in genetically engineered *Streptomyces coelicolor* strains. (A) Pathway in mutants with intact clorobiocin cluster; (B) Pathway in mutants with cloU-defective clorobiocin cluster; (C) Pathway in mutants complemented with pRHAM; (D) Pathway in mutants complemented with pMPU.

aglyca 8 and 9 (Fig. 1), but only small amounts of the desired L-rhamnoside derivatives novclobiocin 122 and 123 (Figs. 1 and 3A).

In clorobiocin biosynthesis, the deoxysugar moiety is formed by five consecutive enzymatic steps (Fig. 1): dTDP-glucose synthetase (CloV), dTDP-glucose-4,6-dehydratase (CloT), 3,5-epimerase (CloW), 5-*C*-methyltransferase (CloU) and 4-ketoreductase (CloS). Most of these reactions are commonly found in the pathways to L-rhamnose and related sugars in various organisms (Salas and Mendez, 2005; Trefzer et al., 1999), but the 5-*C*-methylation by CloU is unusual. When we deleted the gene *cloU* from a cosmid containing the entire biosynthetic gene cluster of clorobiocin and expressed the modified gene cluster in the heterologous host *S. coelicolor* M512, HPLC analysis showed the accumulation of the aglyca 8 and 9, besides smaller quantities of the L-rhamnoside derivative novclobiocin 122 and its structural isomer novclobiocin 123 (Figs. 1 and 3A). The structures of these compounds were unequivocally determined by NMR and mass spectroscopy. In the *cloU*-defective strain, >97% of the aminocoumarins were accumulated in form of aglyca, and only 2.3% in form of glycosides (Freitag et al., 2006). This is in contrast to strains expressing the intact clorobiocin cluster, which produce more than 90% of the aminocoumarins in glycosylated form (Eustáquio et al., 2005).

In clorobiocin and novobiocin biosynthesis, the glycosyltransferases CloM and NovM transfer a 5-*C*-methyl-L-rhamnose (Fig. 1, 6) from dTDP to the coumarin aglycon 9 (Fig. 1) (Li and Heide, 2004). In novobiocin, the