Kirromycin is the main product of the soil-dwelling Streptomyces collinus Tü 365. The elucidation of the biosynthetic pathway revealed that the antibiotic is synthesised via a unique combination of trans-/cis-AT type I polyketide syntheses and non-ribosomal peptide synthetases (PKS I/NRPS). This was the first example of an assembly line integrating the three biosynthetic principles in one pathway. However, information about other enzymes involved in kirromycin biosynthesis remained scarce. In this study, genes encoding tailoring enzymes KirM, KirHVI, KirOI, and KirOII, and the putative crotonyl-CoA reductase/carboxylase KirN were deleted, complemented, and the emerged products analysed by HPLC-HRMS and MS/MS. Derivatives were identified in mutants ΔkirM, ΔkirHVI, ΔkirOI, and ΔkirOII. The products of ΔkirOI, ΔkirOII, and kirHVI were subjected to 2D-NMR for structure elucidation. Our results enabled functional assignment of those enzymes, demonstrating their involvement in kirromycin tailoring. In the ΔkirN mutant, the production of kirromycin was significantly decreased. The obtained data enabled us to clarify the putative roles of the studied enzymes, ultimately allowing us to fill many of the missing gaps in the biosynthesis of the complex antibiotic. Furthermore, this collection of mutants can serve as a toolbox for generation of new kirromycins.