The purpose of this study was to identify and characterize fungal natural products (NPs) with in vitro bioactivity towards leukemia cells. We based our screening on a combined analytical and bio-guided approach of LC-DAD-HRMS dereplication, explorative solid-phase extraction (E-SPE), and a co-culture platform of CLL and stromal cells. A total of 289 fungal extracts were screened and we tracked the activity to single compounds in seven of the most active extracts. The novel ophiobolin U was isolated together with the known ophiobolins C, H, K as well as 6-epiophiobolins G, K and N from three fungal strains in the Aspergillus section Usti. Ophiobolins A, B, C and K displayed bioactivity towards leukemia cells with induction of apoptosis at nanomolar concentrations. The remaining ophiobolins were mainly inactive or only slightly active at micromolar concentrations. Dereplication of those ophiobolin derivatives possessing different activity in combination with structural analysis allowed a correlation of the chemical structure and conformation with the extent of bioactivity, identifying the hydroxy group at C3 and an aldehyde at C21, as indispensable for the strong activity of the ophiobolins. The known compounds penicillic acid, viridicatumtoxin, calbistrin A, brefeldin A, emestrin A, and neosolaniol monoacetate were identified from the extracts and also found generally cytotoxic.