Diastereomeric mixture on the peroxide portion of an endoperoxide acetylmajapolene A (1) was efficiently separated by HPLC on a chiral column, submitting to vibrational circular dichroism (VCD) investigation. The ab initio theoretical VCD and IR calculations of 1a and 1b were performed by density functional theory (DFT) using the B3PW91/6-31G(d,p) level of theory. Focusing on an isolated characteristic peroxide vibrational band, absolute configurations of 1a and 1b were unambiguously determined as (1R,4R,7S,10S) and (1S,4S,7S,10S), respectively. This is the first VCD application to endoperoxides which exist abundantly in nature.