Discovery of new tricyclic spiroindole derivatives as potent P-glycoprotein inhibitors for reversing multidrug resistance enabled by synthetic methodology-based library

Tao Yu,[a, b‡] Rong Zeng,[b, c ‡] Yu Guan,[d‡] Bin Pan,[b] Hong-Wei Li,[b] Jing Gu,[b] Peng-Fei Zheng,*[b] Yan Qian*[a] and Qin Ouyang*[b]

[a] Department of Pharmacy, The Second Affiliated Hospital of Chongqing Medical University, Chongqing,400010, China

[b] Department of Medicinal Chemistry, Third Military Medical University, Shapingba, Chongqing 400038 China

[c] Department of Gastroenterology, Xinqiao Hospital, the Second Affiliated Hospital of Army Medical University(Third Military Medical University), Chongqing 400037, Chinad

[d] College of Chemistry and Environmental Engineering, Sichuan University of Science and Engineering, Zigong, 643000, China.

‡ These authors contributed equally.

E-mail: ouyangq@tmmu.edu.cn, cqqianyan@hospital.cqmu.edu.cn, pengfeiz@tmmu.edu.cn

Supporting Information

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1. Phenotypic screening

Compounds obtained from synthetic research were used to establish a compound library containing compounds with different skeletal structures. Based on the research model of synthetic methodology, organic synthesis research has provided many compounds with high molecular complexity, where each type of compound skeleton has generated many structurally similar derivative compounds; similar to the lead compound structure optimization methods in new drug development. In our research group's previous work, this solid compound library was utilized successfully using a specific drug screening model to screen GIT/PIX[1]protein interaction inhibitors.

In this study, the ability of P-gp inhibitors to inhibit the efflux of specific P-gp fluorescent substrates within cells was evaluated, and P-gp inhibitors were identified through a simple experimental procedure. The initial screening involved selecting 2-4 compounds with the same skeletal structure from the physical compound library. Fortunately, compounds with different skeletal structures exhibited varying levels of inhibitory activity. Subsequently, more experiments were conducted to reverse MDR using compounds structurally similar to **7** (**S25** in table S1) and **14** (**S26** in table S1). Further research on the highly active **S62-S67** series of compounds was not carried out due to the potential health risks associated with the aldehyde groups present in these compounds.

Rhodamine 123[2, 3]dye is a membrane-permeable cationic fluorescent probe that specifically recognizes mitochondrial membrane potential, thereby binding to mitochondria and producing bright fluorescence. At certain concentrations, rhodamine dye has low toxicity to cells, making it commonly used for detecting mitochondria in animal cells, plant cells, and microorganisms. Rhodamine 123 is also a P-gp-specific fluorescent substrate. When rhodamine 123 enters the cell, it is effluxed by P-gp on the cell membrane, reducing the residual dye inside the cell. In the presence of P-gp inhibitors, this efflux process is significantly reduced, leading to increased fluorescence intensity inside the cell. Our screening model utilizes P-gp inhibitors to inhibit the efflux of specific P-gp fluorescent substrates within cells, enabling rapid and accurate screening of P-gp inhibitors as detailed in the following text.

Eca109/VCR cells overexpressing the drug-resistant protein P-gp were seeded onto a black 96-well plate (15000 cells per well) and cultured for 12 hours. Compounds or positive controls (VRP, TQ) were treated at a concentration of 25 μM for 0.5 hours, followed by the addition of rhodamine 123 (final concentration 5 μM) for 2 hours. After treatment, the culture medium was aspirated, and each well was washed three times with pre-cooled PBS. The total fluorescence intensity of each well was measured by a Multi-Mode Detection Platform (SpectraMax Paradigm, olecular Devices, USA), and the data were analyzed using GraphPad Prism 8.

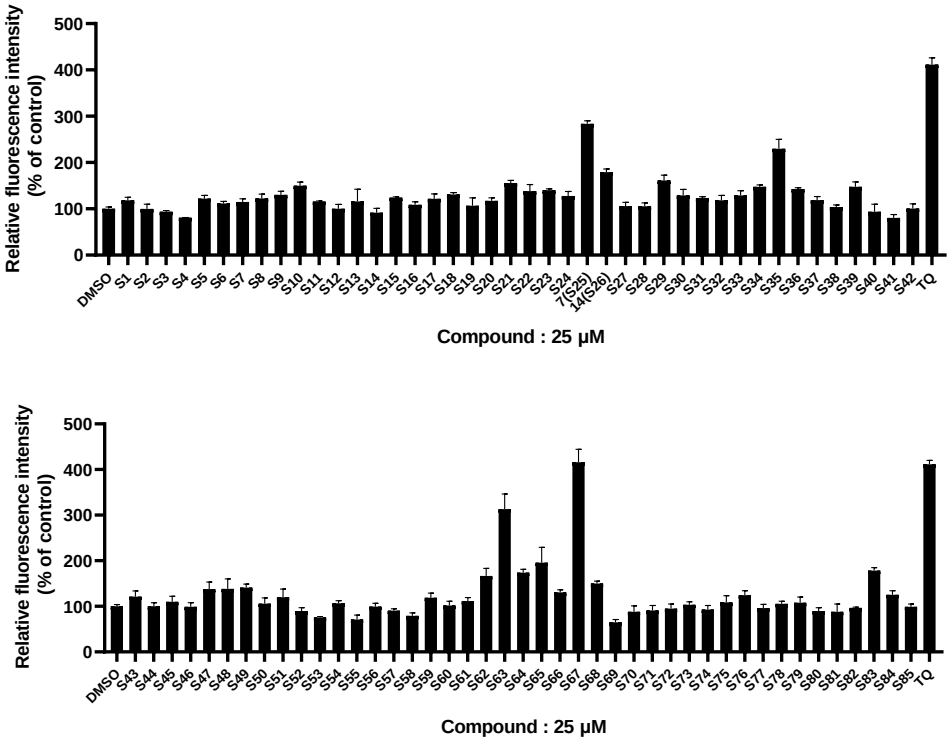


Figure S1 Effect of Compounds (25 μM) on the accumulation of Rhodamine 123 in Eca109/VCR cells.

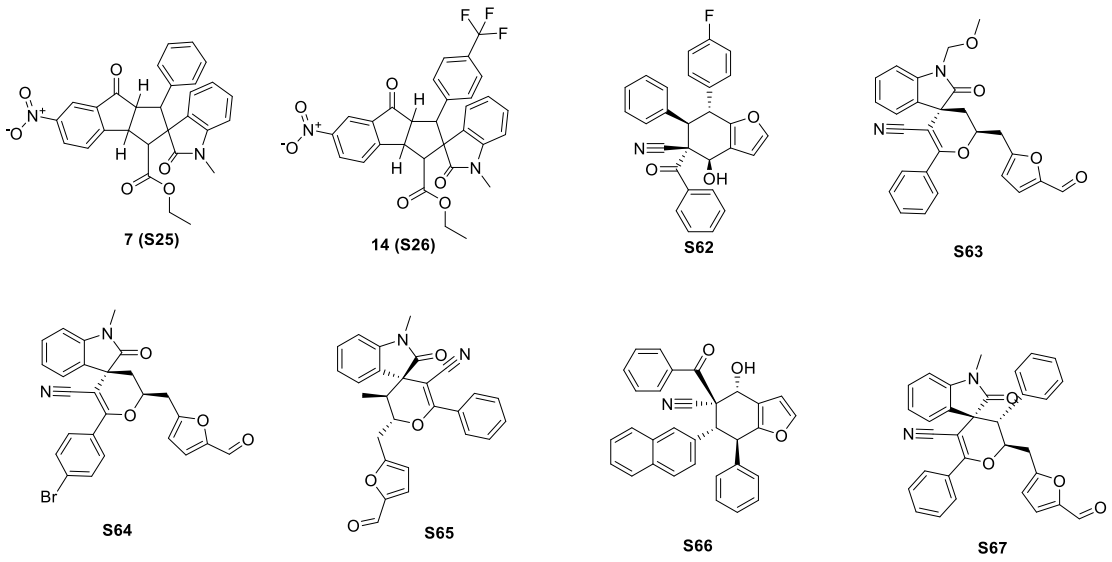


Figure S2 Partial structures of tested compounds (**S25-S26 and S62-S67**)

2. Spectra of OY-103 (NMR, HRMS and HPLC)

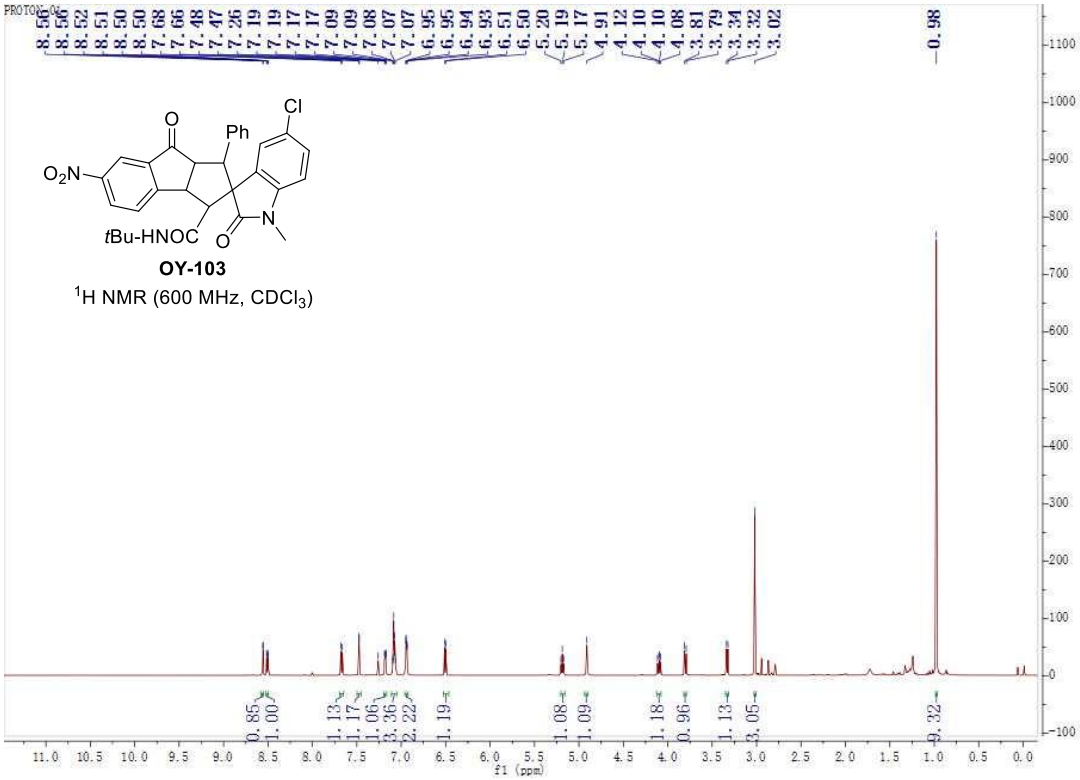


Figure S3 ^{1}H-NMR spectra of **OY-103**

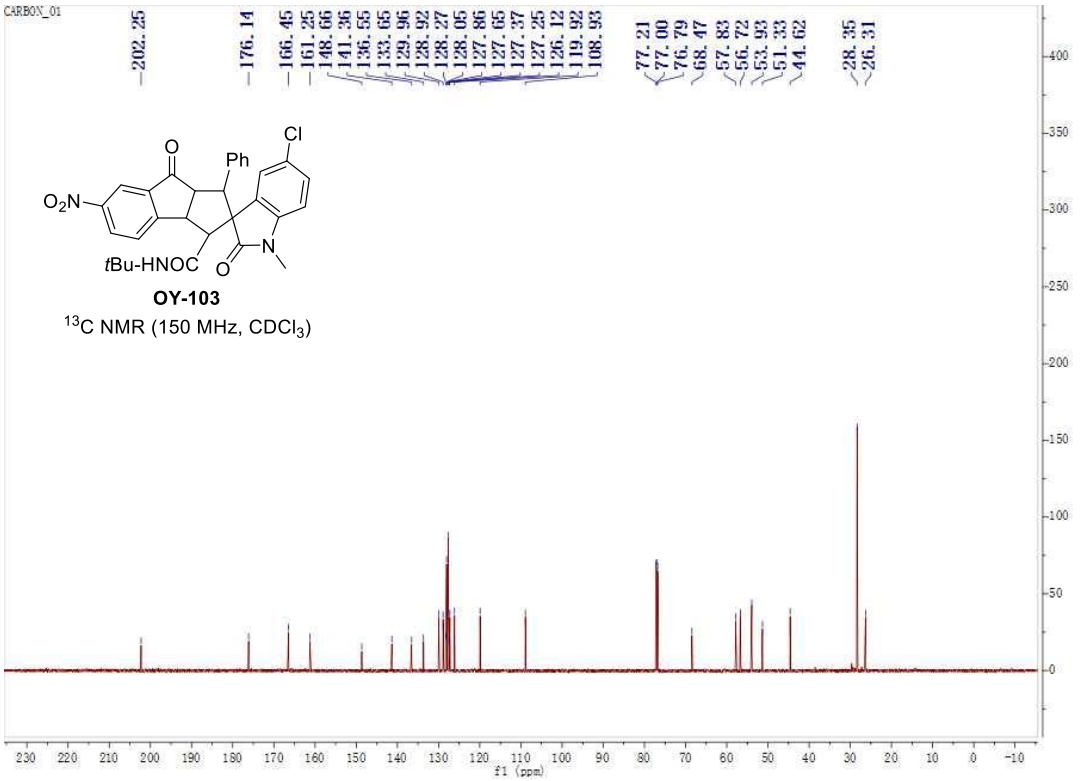


Figure S4 ^{13}C-NMR spectra of **OY-103**

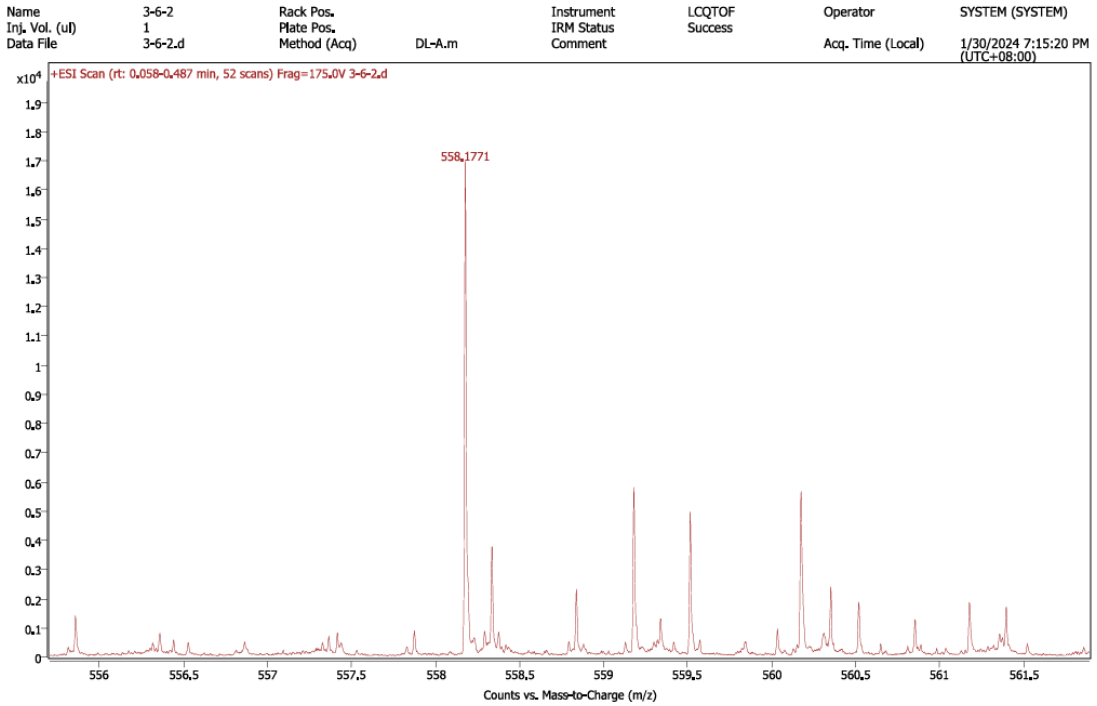


Figure S5 HRMS spectrum of **OY-103**

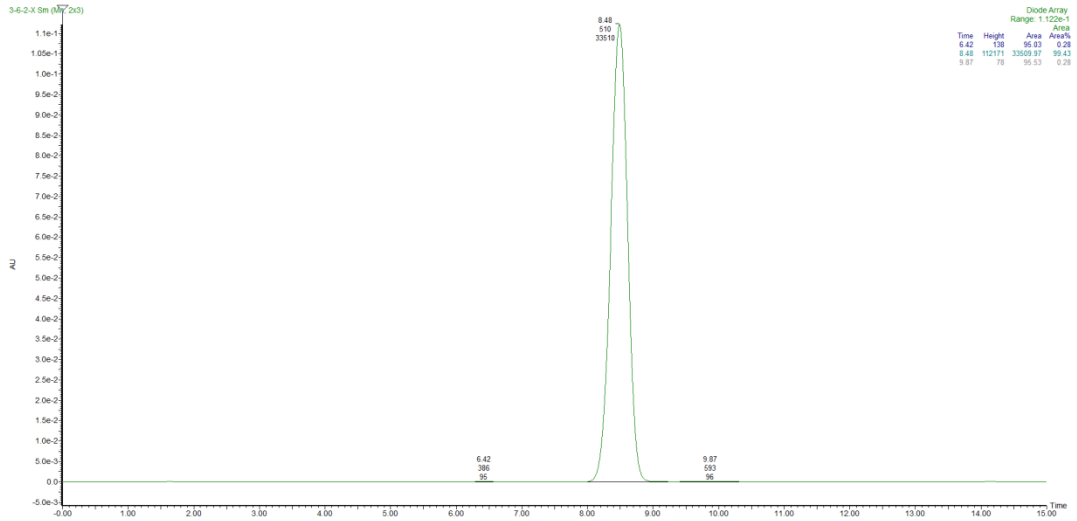


Figure S6 HPLC spectrum of racemic **OY-103** (Waters e2695 with Shim-pack GIST C18 reversed-phase column (4.6 mm ×250 mm, 1.7 μm, H_2O : MeOH = 40 : 60, flow rate = 1.0 mL/min).)

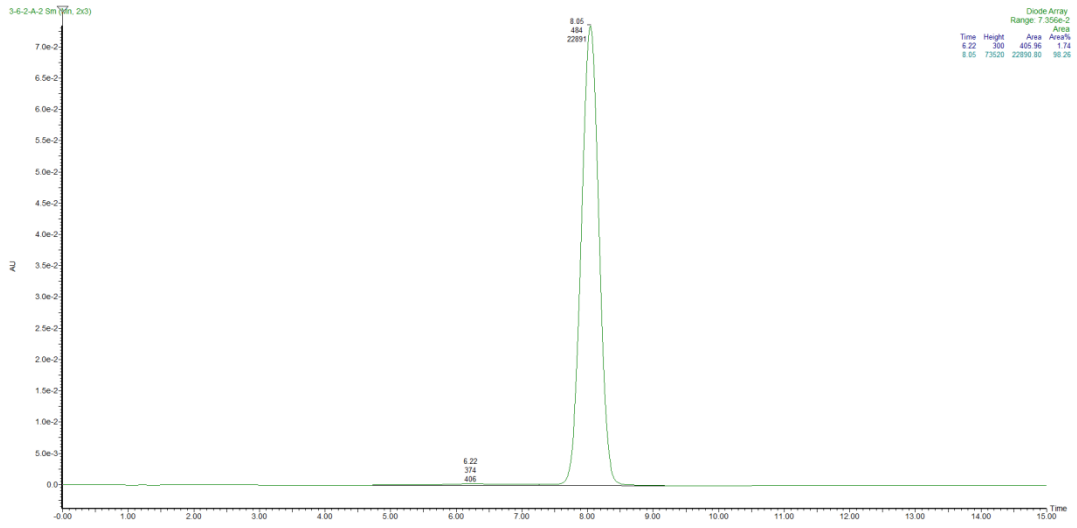


Figure S7 HPLC spectrum of **OY-103-A** (Waters e2695 with Shim-pack GIST C18 reversed-phase column (4.6 mm ×250 mm, 1.7 μm, H_2O : MeOH = 40 : 60, flow rate = 1.0 mL/min).)

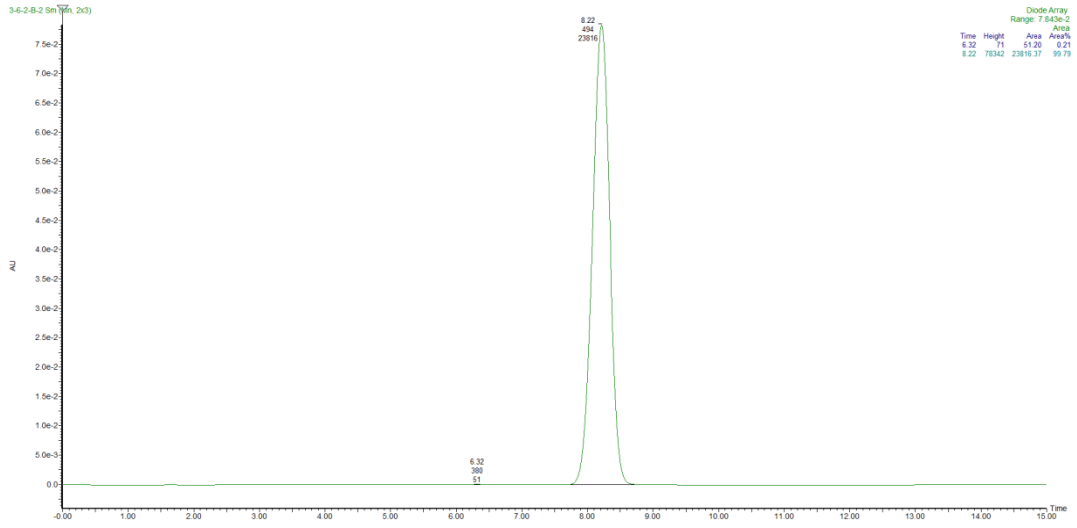
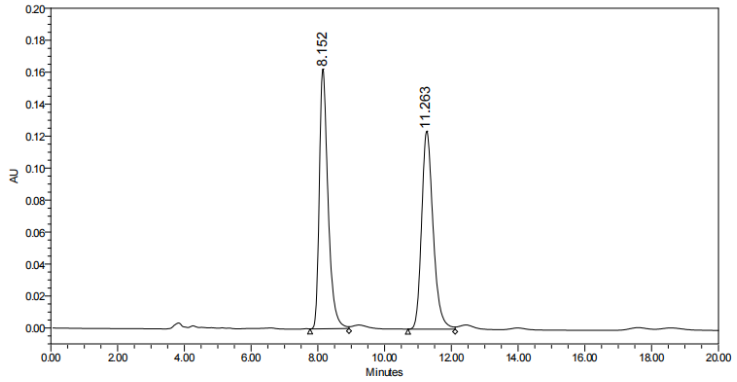
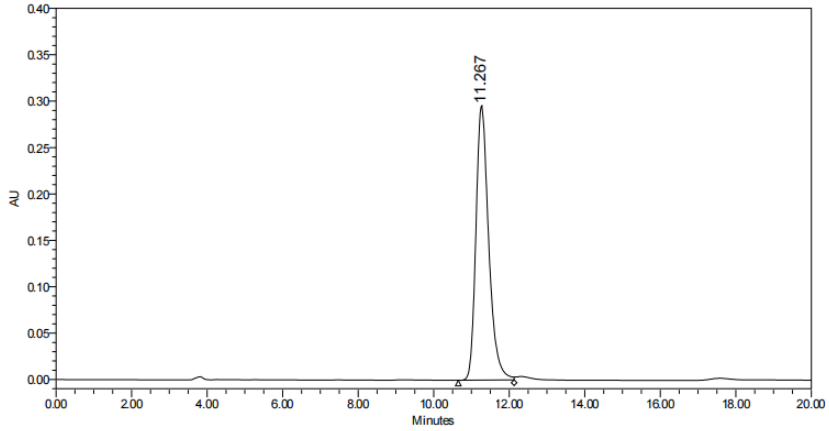


Figure S8 HPLC spectrum of **OY-103-B** (Waters e2695 with Shim-pack GIST C18 reversed-phase column (4.6 mm ×250 mm, 1.7 μm, H_2O : MeOH = 40 : 60, flow rate = 1.0 mL/min).)



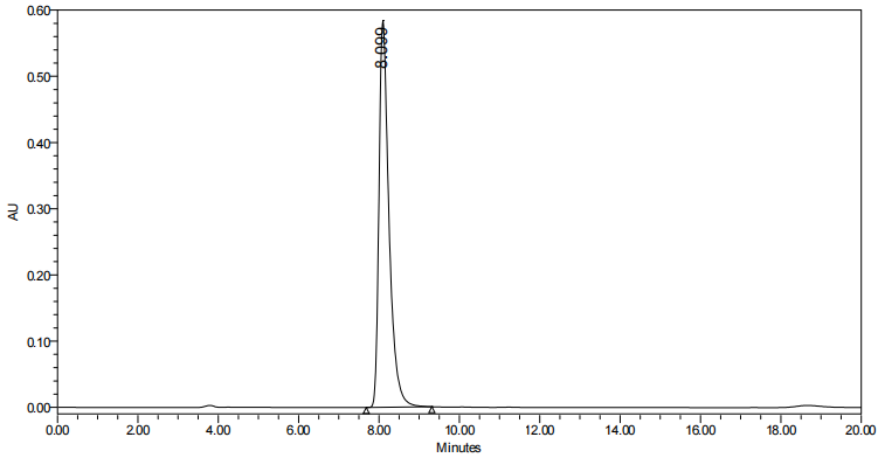
	RT	Area	% Area	Height
1	8.152	2941847	49.72	162553
2	11.263	2974841	50.28	123951

Figure S9 Chiral HPLC spectrum of racemic **OY-103** (The stationary phase was Daicel chiral IA column and the mobile phase consisted of a mixture nHexane/iPrOH (60/40) delivered at a flow rate of 0.5 mL/min under 25 ºC)



	RT	Area	% Area	Height
1	11.267	6954581	100.00	295662

Figure S10 Chiral HPLC spectrum of racemic **OY-103-A** (The stationary phase was Daicel chiral IA column and the mobile phase consisted of a mixture nHexane/iPrOH (60/40) delivered at a flow rate of 0.5 mL/min under 25 ºC)



	RT	Area	% Area	Height
1	8.099	10120621	100.00	584169

Figure S11 Chiral HPLC spectrum of racemic **OY-103-B** (The stationary phase was Daicel chiral IA column and the mobile phase consisted of a mixture nHexane/iPrOH (60/40) delivered at a flow rate of 0.5 mL/min under 25 ^{o}C)

References

1. J. Gu, R.-K. Peng, C.-L. Guo, M. Zhang, J. Yang, X. Yan, Q. Zhou, H. Li, N. Wang, J. Zhu and Q. Ouyang, *Nat. Commun.*, 2022, **13**, 7176.
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