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Crystal and molecular structures of a natural equimolecular mixture of two epimeric diterpenes

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Abstract—Irroratin A (**1**), isolated from the aerial parts of *Isodon irrorata* (Forrest.) (Labiatae), was shown to be an equimolecular mixture of two C-20 epimers of a new *ent*-kaurene diterpene both in the crystalline state and pyridine solution, based on X-ray, LC/MS/MS, FABMS as well as extensive 1D- and 2D-NMR spectral analysis. The two epimers were bound together by hydrogen bonds, and when in chloroform and methanol solution, the 20*S*-epimer predominates. Irroratin A exhibited potent cytotoxicity against several human cancer cell lines.

1. Introduction

Isodon (Labiatae) is a genus comprising 150 species, with more than 100 being distributed in China. Some 10 species have been used in Traditional Chinese Medicine (TCM) for the treatment of gastrointestinal disorders and more recently as antitumor agents.[1,2] Our previous phytochemical studies on the plants of this genus have led to the isolation of more than 200 novel *ent*-kaurene diterpenes, some of which showed potential cytotoxic activity in vitro.[3] As part of our continuing search for novel chemical structures with cytotoxicity against human cancer cells, *Isodon irrorata* (Forrest.) Hara, a plant growing mainly in northwestern Yunnan Province, China, was chosen for phytochemical study since there is no reported phytochemical work on this plant. From the stems and leaves of this plant, a mixture of two unique diterpene epimers, which was collectively named irroratin A (**1**), and the known flavonoid glycoside rutin were isolated. This report describes the isolation, structure determination and cytotoxicity of of irroratin A (**1**).

2. Results and discussion

Compound **1** displayed a single spot on TLC (silica gel) developed in several solvent systems and its homogeneity was confirmed by its NMR spectral behavior. However, the ^{1}H- and ^{13}C NMR spectra taken in pyridine-d_5 appeared to be unduly complex. A closer inspection of the NMR spectra revealed its appearance as being an equimolecular 'mixture' of structurally-related components as evidenced by the presence of two sets of equal intensity ^{1}H NMR signals.

2.1. Crystal structure of irroratin A (1)

Crystalline plates suitable for X-ray crystallographic analysis were grown in methanol solution. The crystal structure of **1** was indeed that of a mixture of two diastereoisomers, epimeric at the C-20 hemiacetal hydroxyl function. The two moieties were bound by intermolecular hydrogen bonds. It appeared that the two molecules in the asymmetric unit differ only in the configurations at the C-20 group. As shown in Fig. 1, the molecules in the crystals have a disordered OH group at the C-20 position. As shown in Fig. 2, there are two intermolecular H-bonds, i.e. O4′–O_2a (2.678 Å) and O5′–O_2b (2.784 Å), connecting the symmetrical molecules.

Keywords: acetals; terpenoids; *isodon irrorata*; complexes; epimerisation.
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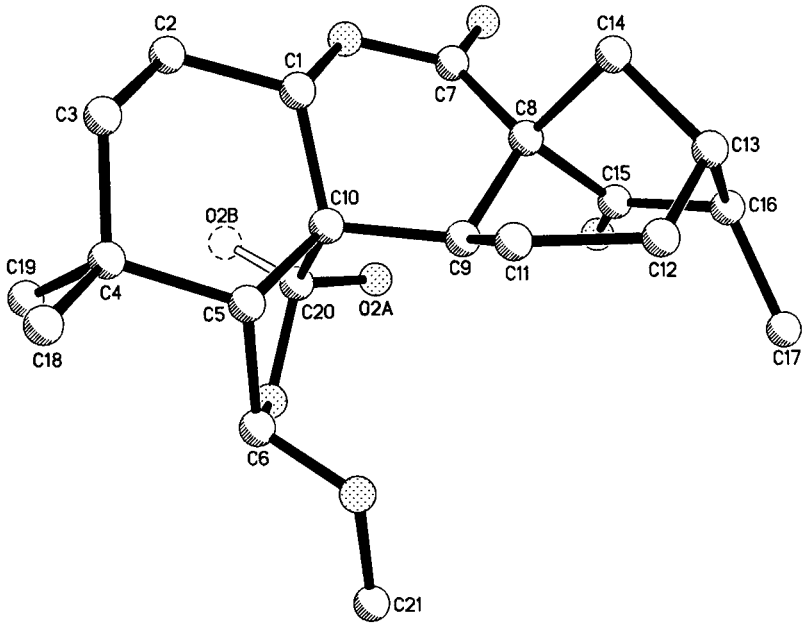


Figure 1. Computer-generated perspective drawing of the final X-ray model of irroratin A (**1**).

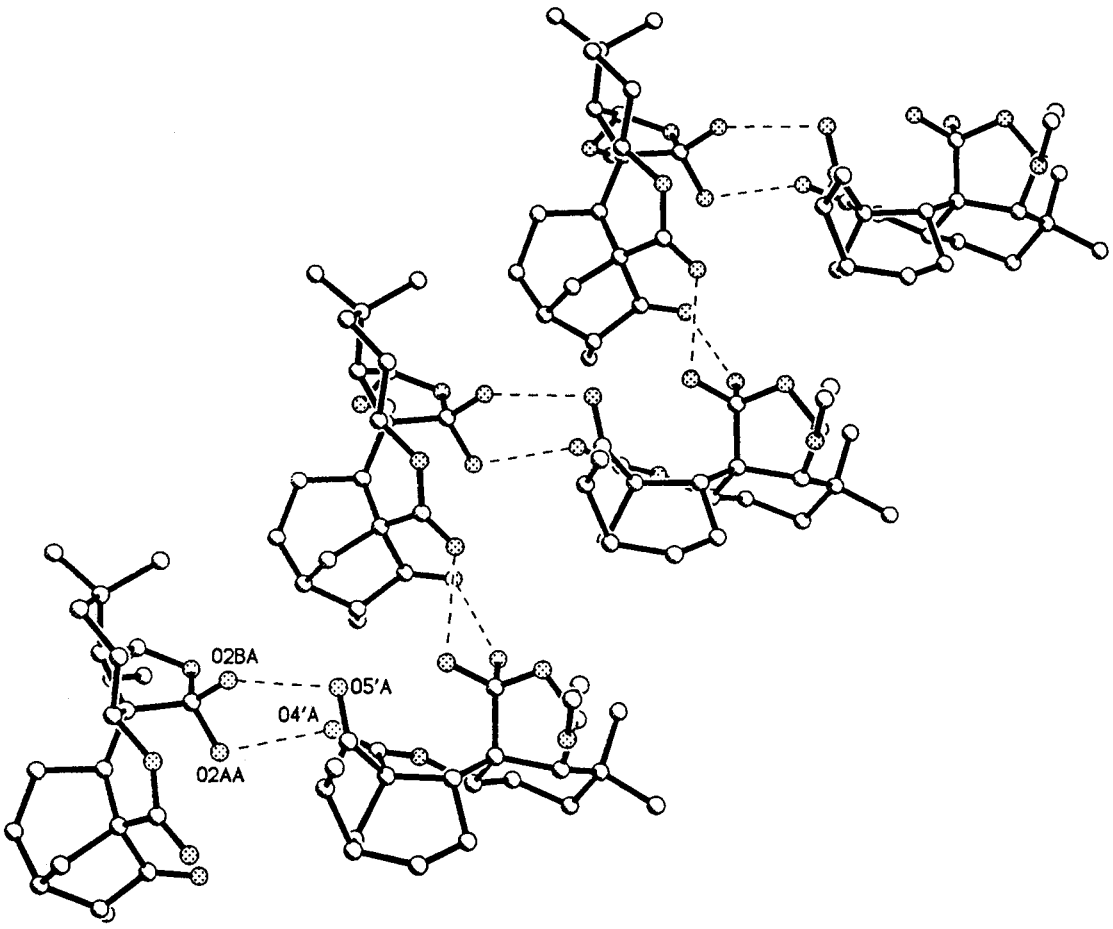


Figure 2. Crystal structure irroratin A (**1**) viewed from the crystallographic C-axis. Dished lines represent hydrogen bonds.

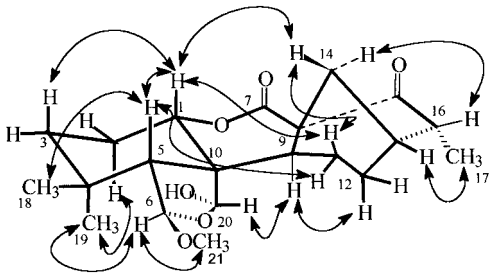
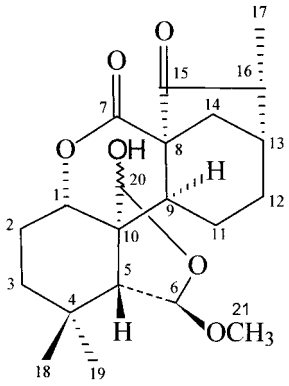


Figure 3. The key ROESY correlations of 20*R*-epimer.



Irroratin A (**1**)

It is well-known that organic molecules with similar size, shapes and functionalities have similar crystal structures and tend to form mixed crystals (cocrystals) and solid solution.[4,5] In most cases, the components in the mixed crystal or solid solution are randomly distributed on the lattice sites. Consequently, no superstructure could be detected, such as in the cases of mixed crystals of cyclopentanones[4] and cocrystals of (−)-podopetaline and (−)-ormosanine.[6] The driving forces for this kind of cocrystal formation is the crystal packing and the optimized van de Waals interactions resulting from mutual matching surfaces. Also encountered, but less frequently, are so-called complexes, such as melamine-cynuric acid cocrystal;[7] a 1:2 complex of 1, 4-dinitrobenzene and 4-iodocinnamic acid;[5] a 1:1 complex of 1, 4-dinitrobenzene and 1:4-diiodolenene;[5] and the 1:1 complex of 6-hydroxydopamine hydrochloride and its oxidized form, *p*-quinonoid.[8]

In view of the above, it is not unexpected that natural products have been isolated as mixtures of epimers. For example, C-27 steroids (and their glycosides) are frequently found in epimeric pairs at either the C-25 or the C-22 positions.[9,10] Other examples include prenylated flavonoids,[11] and diterpenes and acetogenins involving a hemiacetal functionality.[12,13]

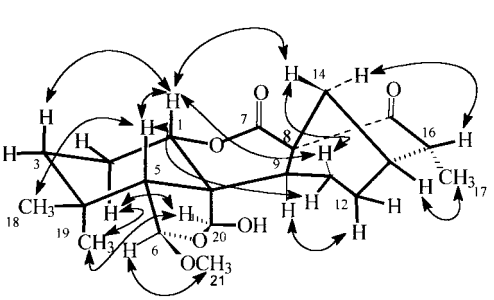


Figure 4. The key ROESY correlations of 20*S*-epimer.

Recently, we reported the isolation of DCRA, a well-defined 1:1 complex of the diterpenes neoangustifolin and epinodosinol from *Rabdosia* (*Isodon*) *angustifolia*. In that case, the two different substrates were bound together by an intermolecular hydrogen bond and the very good hydrophobic close approach of mutual matching surfaces.[14]

Therefore, by analogy with the ongoing example, the crystal structure of **1** was either a solid solution or a complex of two C-20 epimers stabilized by hydrogen bonding and crystal packing.

2.2. Electrospray and FAB mass spectroscopy of irroratin A (1)

Electrospray ionisation has become an important technique in mass spectroscopy in the last few years. As ions are generated directly from aqueous solution with only mild heating, electrospray ionization mass spectroscopy (ES-MS) is suitable for the study of intermolecular interactions and molecular recognition.[15]

ESMS has also been frequently employed in the field of supermolecular chemistry for the investigation of synthetic host-guest systems, since ionization of the targeted molecular species occurs under very mild conditions and fragmentation is usually not observed in electrospray MS experiments, which suggests that non-covalent molecular association complexes might be detectable.[16–18] Evidence for the formation of tight complexes of **1** in the solid state comes from the electrospray MS/MS and FAB mass spectrometry. In the positive mode electrospray MS/MS spectra of **1**, the dimeric complex adduct parent ion was detected at *m/z* 779 $[2M+Na]^+$, which liberated daughter ions at *m/z* 401 $[2M+Na-378]^+$ and *m/z* 346 $[2M+Na-378-CH_3OH]^+$. The fragmentation pattern is in good agreement with the dimeric complex characters of **1**. Moreover, the FAB mass spectrum gave further supporting evidence for the presence of a complex,[19] in which a prominent adduct parent ion was observed at *m/z* 757 $[2\times M+H]^+$, in addition to the base peak at *m/z* 707 $[2\times M+H-18-CH_3OH]^+$, corresponding to the loss of one molecule of water and one molecule of methanol. Other ions were detected at *m/z* 379 [monomer molecular ion+1]$^+$ and 361 [monomer parent ion+1-18]$^+$.

2.3. Structure of irroratin A (1) in pyridine-d_5 solution

There are two well-resolved sets of signals of equal intensity observed in both the ^1H and ^{13}C NMR spectra taken in pyridine-d_5, which suggested that the two epimers existed in equal populations in pyridine solution.

Despite the highly complex nature of the NMR spectra of **1** due to the structure similarity and proximity of the chemical shifts of the two epimers in the complex, the NMR assignments of **1** could still be achieved by employing a combination of 1D- and 2D-NMR techniques, including

Table 1. ^{1}H and ^{13}C NMR assignments of the two epimers of irroratin A (**1**) (recorded in C_5D_5N, chemical shift values were reported as δ values (ppm) from internal TMS at 500 MHz, signal multiplicity and coupling constants (Hz) are shown in parentheses)

	^{1}H		^{13}C	
Carbon	20*S*-	20*R*-	20*S*-	20*R*-
1	4.56 (dd, 5.5, 11.5)	4.58 (t, 8.5)	77.21	76.26
2	2.93 (m); 1.77 (m)	1.79 (2H, m)	24.93	23.78
3	β:1.46 (dt, 4.5, 12.5); α:1.22 (m)	β:1.30 (dt, 4.0, 12.5); α: 1.15 (m)	38.16	37.49
4			31.33	31.22
5	1.99 (br.s)	2.07 (br.s)	53.46	54.19
6	4.68 (br.s)	5.13 (br.s)	105.33	107.75
7			172.39	172.88
8			57.20	56.21
9	2.09 (dd, 5.0, 12.5)	2.92 (dd, 6.0, 12.5)	45.76	38.75
10			51.39	50.13
11	β: 1.38 (m); α: 1.79 (m)	β:1.36 (m); α: 1.77(m)	19.35	18.71
12	β: 1.36 (m); α: 1.68 (m)	β:1.33 (m); α:1.74 (m)	20.21	19.26
13	2.38 (m)	2.27 (m)	32.79	32.10
14	α: 2.55 (d, 12.5) β: 1.84 (dd, 3.0, 12.5)	α: 2.51 (d, 11.5) β: 1.93 (dd, 4.0, 11.5)	34.74	35.14
15			215.53	212.42
16	2.29 (m)	2.35 (m)	49.75	49.27
17	0.98 (d, 6.5)	0.95 (d, 6.5)	10.79	10.79
18	1.00 (s)	0.92 (s)	33.84	32.65
19	1.42 (s)	0.90 (s)	22.99	23.52
20	6.12 (s)	6.07 (d, 4.5) 9.43 (d, 4.5, OH)	101.31	105.25
21	3.21 (s)	3.18 (s)	54.76	54.71

DQF-COSY, HOHAHA, HMQC, HMBC and ROESY experiments. Since the two monomers of **1** differ only in the configuration of the C-20 hydroxyl group, the H-20 signals, resonating at the lowest field, were selected as the starting point for the NMR assignments of each monomer. In the ROESY spectrum of **1** (as summarized in Figs. 3 and 4), the doublet signal for H-20 at δ 6.07, which was coupled with the 20-OH group from DQF-COSY spectrum, was ascribed to the 20*R*-epimer based on the informative ROE correlation between H-20 with a methine signal at δ 2.92, assignable to H-9, rather than H_3-19 (δ 0.90). It was very clear that this assignment was possible only with a 20*R*-configuration on inspecting the Dreiding molecular model. Consequently, its epimeric counterpart, a singlet signal at δ 6.12, should be assigned to H-20 of the 20*S*-epimer, which showed ROE cross peaks with H_3-19 at δ 1.42 and H-2α at δ 1.77, as expected from the molecular model. The proton H-2α of the 20*S*-epimer provided a starting point to assign the signals of H-1, H-2β and H-3 through the information derived from DQF-COSY and the HOHAHA spectra. Also, the assignment of C-1, C-2 and C-3 could be accomplished from the HMQC experiment. The corresponding NMR signals for the 20*R*-epimer were similarly assigned starting from H-9. Finally, the HMBC experiment was employed for the assignment of the quaternary carbons, in addition to providing confirmation of the established carbon–carbon connectivities. Thus, the ^{1}H- and ^{13}C NMR spectral data of the two epimers were completely and unambiguously assigned (Table 1).

2.4. Acetylation of irroratin A (1)

The presence of the hemi-acetyl group at the C-20 can be expected to lead to epimerization in aqueous solution, as often happens in the cases of aldehyde–sugars (aldoses). This effect was confirmed by the acetylation of compound **1** with Ac_2O/pyridine, which afforded a single product identified as 20*S*-acetylirroratin A by NOESY spectroscopy, in which a conclusive NOE correlation between H-20 and H_3-19 was observed. The fact that only one product was formed could be explained by the observation that in the Dreiding model the 20*R*-OH lies in a sterically hindered environment, whereas the 20*S*-OH is less hindered, and thus it is easier for acetylation to occur.

2.5. Molecular structures of irroratin A (1) in MeOH-d_4, DMSO-d_6 and $CDCl_3$

In order to obtain more detailed insight about the solution structures of **1**, its NMR spectra were measured in different solvents. As a result, it was found that the population of the epimers and conformations of **1** were very sensitive to the solvent in which it was dissolved, similar to the cases of γ-hydroxybutenolides isolated from marine sponge.[20] In DMSO-d_6, the two epimers existed at equal abundance. In MeOH-d_4 solution, the ratio of the 20*S*-and 20*R*-epimers is 3:2 based on the integration of the ^{1}H NMR spectrum, while in $CDCl_3$, more than 85% is the 20*S*-epimer as evident from the ^{1}H NMR and ROESY spectra, in which an informative cross peak between H_3-19 and H-20 was observed in the ROESY spectrum, suggesting that the C-20 configuration is *S*. After the removal of $CDCl_3$ solvent by continuous purging with nitrogen, the two epimers returned to the 1:1 ratio in pyridine-d_5 solution as evident from ^{1}H NMR spectroscopy.

Irroratin A was evaluated for cytotoxicity using human cancer cell lines according to the published method.[21] It exhibited potent cytotoxicity against Lu-1 (human lung cancer) and LNCap (hormone-dependent human prostate cancer) cell lines at the ED_{50} values of 2.4 and 1.8 μg/ml, respectively.

3. Conclusion

In the current investigation, a novel epimeric diterpene pair,

irroratin A (**1**), was isolated from the stems and leaves of *Isodon irrorata*. Its structure in the crystalline state was unambiguously determined by X-ray crystallography. Compound **1** was shown to have different epimeric ratios and conformations in MeOH-d_4, C_5D_5N, DMSO-d_6 and $CDCl_3$ solutions. This isolate adds to the list of natural products which have been isolated as inseparable mixture of epimers, which is becoming more and more common. Nevertheless, the structure of compound **1** has some notable features. Firstly, in both the solid crystalline form and in pyridine solution, the two epimers are of equal abundance, as determined by X-ray crystallography and NMR spectroscopy. Secondly, it is the first *ent*-kaurene diterpene with hemiacetate functinality at C-20 position.

4. Experimental

4.1. General procedures

Mps: Uncorrected.; IR: film (KBr); ^{1}H- and ^{13}C NMR, DEPT, HMQC, DQF-COSY, HOHAHA and ROESY spectra were recorded on Bruker Avance DRX-500 instrument with TMS as int. standard, using Bruker standard programs. FAB-MS was recorded by the direct-inlet method on a VG ZAB-HS mass spectrometer using glycerol as matrix. Electrospray MS/MS was performed on a Macro mass Quattro II electrospray triple quadrupole mass spectrometer.

4.2. Plant material

The leaves and stems of *Isodon irrorata* (Forrest.) Hara, were collected in September, 1991, at Lijiang Yulong Maintain, Yunnan Province, China, and identified by Professor H.-W. Li of the Kunming Institute of Botany, Chinese Academy of Sciences, China. A voucher specimen was retained at the Herbarium of the Department of Taxonomy, Kunming Institute of Botany.

4.3. Extraction and isolation

The air-dried and milled stems and leaves (2100 g) of *Isodon irrorata* were extracted by maceration with MeOH (4 l×4) and the residue was concentrated under vacuum to afford a gum, which, after dilution a small volume of water, was partitioned with petroleum ether and ethyl acetate, successively. The resulting ethyl acetate extract (45 g) was first absorbed on silica gel (120 g) and then added to a column of silica gel and chromatographed using a mixture of $CHCl_3$ and acetone (5–50%). Work-up of the fractions eluted with 5% acetone–$CHCl_3$ resulted in isolation of 1 as colorless needles (165 mg, 0.0078%) following recrystallization from MeOH. The known flavonoid glycoside, rutin (5.0 g) was obtained from the fraction eluting by 50% acetone–$CHCl_3$ mixture.

4.3.1. Irroratin A (1). Colorless needles from MeOH; mp 185–7°C; $[\alpha]_D=-194.8°$ (*c* 0.4, MeOH). IR (KBr) νmax (cm^{-1}): 3300 (OH), 1760, 1720, 1230, 820. EIMS *m/z* (rel. int. %): $[M]^+$ 378 (15), 350 (25), 348 (18). FABMS (positive) *m/z*: 757 $[2M+H]^+$, 739 $[2M+H-H_2O]^+$, 709 $[2M+H-H_2O-OCH_3]^+$, 379 $[M+H]^+$, 361 $[M+H-H_2O]^+$; (negative): 755 $[2M+H]^-$, 377 $[M-H]^-$, 345 $[M-H-OCH_3]^-$. ES-MS (positive) *m/z*: 779 $[2M+Na]^+$, 401$[M+Na]^+$, 401$[M+Na-CH_3OH]^+$. ^{1}H- and ^{13}C NMR (500 MHz, C_5D_5N) data are presented in Table 1.

4.4. X-Ray crystallography of irroratin A (1)

Colorless plate-like crystal, Triclinic, space group P1, with a=8.7216 (2) Å, b=9.2219 (2) Å, c=13.0036 (4) Å, α=93.759 (**1**)°, β=106.504 (**1**)°, γ=90.121 (**1**)°, V=1000.39 (4) Å^3, Z=2. Data collection was carried out at room temperature on a Bruker SMART CCD-based X-ray diffractometer (λ=0.71073 Å). A total of 2560 frames were collected with a scan width of 0.3° in ω, and an exposure time of 10 s/frame. The structure was solved and refined using the Bruker SHELXTL (version 5.0) Software Package. The final anisotropic full-matrix least-squares refinement on F^2 converaged at R_1=5.64%, wR_2=12.41% and a goodness-of-fit of 0.981.

The atomic coordinates, equivalent isotropic distropic displacement parameters, lengths, bond angles, anisotropic displacement parameters, H-atom coordinates and isotropic displacement parameters of compound **1** (coded 'Lu-2') have been deposited at Cambridge Crystallography center, UK.

4.5. Acetylation of irroratin A (1)

A solution of 1 (5 mg) in pyridine (3 ml) and Ac_2O (6 ml) was kept standing at room temperature over night, work-up of the resulting reaction resulted in the isolation of 20*S*-acetylirroratin A, EIMS *m/z* (rel. int. %): 377$[M-Ac]^+$ (55); DCI *m/z* (rel. int. %): 361$[M+H-AcOH]^+$ (100).

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