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Short communication

The total synthesis of fukiic acid, an HIV-1 integrase inhibitor

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Abstract

A successful synthesis of fukiic acid is described in 7% overall yield (6 steps from veratraldehyde). *rac*-Fukiic acid was found to be a potent inhibitor of HIV-1 integrase but did not reveal any antiviral activity in the MT-4 cells assay.

Keywords: Natural polyphenol; Anti-HIV-1; Integrase inhibitors

1. Introduction

In 1969, a new polyphenol, Fukinolic acid, was isolated from *Petasites Japonicus* by Sakamura et al. [1]. Its alkaline hydrolysis gave caffeic acid and a new polyphenol, named fukiic acid. Matsumoto et al. [2] proposed the structure of fukiic acid and Yoshihara et al. [3,4] determined that fukiic acid has the same stereochemical features as piscidic acid as (2*S*,3*R*)-2,3-dihydroxy-4-(3,4-dihydroxyphenyl)-3-carboxybutyric acid. Several other rare hydroxycinnamic acid esters, such as cimicifugic acids, fukinolic acids and fukiic acid, were also isolated from some *Cimicifuga* species [5].

Black cohosh (*Cimicifuga racemosa*) is a North American perennial in the buttercup family (Ranunculaceae) that has been in use in Europe for the last 50 years as a natural alternative to hormone replacement therapy for women with breast cancer. *C. racemosa* extract inhibits proliferation of MCF-7 and MDA-MB231 breast cancer cells by induction of apoptosis [6,7]. Amongst the different components of the extract are the triterpene glycosides responsible for the growth inhibitory activity and specially actein. The latter has also revealed anti-HIV activity [8]. Other constituents are the phenolic esters that have no effect on the proliferation of MCF-7 cells [9].

The structural similarities with the known HIV-1 integrase inhibitor [10], chicoric acid (Scheme 1) prompted us to synthesize *rac*-fukiic acid **1** and evaluate its antiviral properties. Indeed, fukiic acid also possesses a tartaric acid moiety and a 3,4-dihydroxyphenyl group. Tartaric acid and catechol are known to efficiently bind magnesium ion [11], suggesting an expected activity as HIV-1 integrase inhibitor via the interaction with the magnesium ion(s) present in the active site of this enzyme. However, this mechanism has not been fully established yet. The synthesized *rac*-fukiic acid was effectively found to be active against the reactions catalyzed by HIV-1 integrase but inactive against the viral replication in MT-4 cell assay. We present herein the synthesis and the biological properties of this new IN inhibitor.

2. Results and discussion

2.1. Chemistry

The synthesis of *rac*-fukiic acid dimethyl ether **2** was previously briefly described in 1972 [2] and we reinvestigated all

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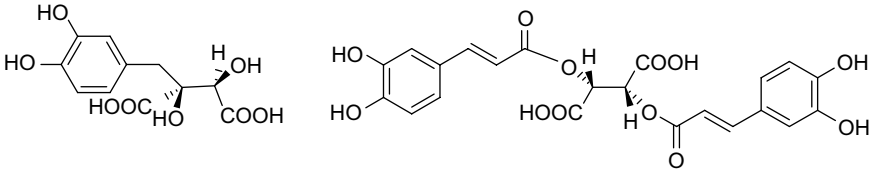


Fukiic acid L-Chicoric acid

Scheme 1.

the steps until the final deprotection leading to the target molecule that had never been addressed (Scheme 2). The synthesis starts by the conversion of veratraldehyde **3** into the oxazolone **4** by the Erlenmeyer reaction [12]. The acidic hydrolysis of **4** affords the pyruvic acid **5** [12] which is esterified using methyl iodide and DBU in dry DMF giving **6** [13]. Wittig reaction of this pyruvic acid methyl ester **6** with methoxycarbonylmethyltriphenylphosphorane quantitatively affords a mixture of dimethyl 3,4-dimethoxyphenylmethylmaleate **7** as the major isomer (75%) and dimethyl 3,4-dimethoxyphenylmethylfumarate **8** (25%) that can be quantitatively separated by column chromatography. The *Z* stereochemistry of **7** was assigned according to the characteristic chemical shifts of the methylenic (3.58 ppm in $CDCl_3$) and ethylenic (5.63 ppm) proton signals and their cisoïd coupling constant ($^4J = 1.9$ Hz), the *E* stereochemistry of **8** being attested by the downfield shift of the methylenic proton signal (3.72 ppm), the high field shift of the ethylenic proton signal (4.12 ppm) and the absence of an observable transoïd coupling constant. The *Z* and *E* stereochemistries of **7** and **8** were also confirmed by NOESY experiments. Compound **7** is then oxidized using potassium permanganate and magnesium sulphate in ethanol at 0 °C in *rac*-fukiic acid dimethyl ether **2** in 68% yield. The same reaction on **8** gives **9**, the diastereoisomer of **2** in 75% yield. The critical step in the synthesis of *rac*-fukiic acid is the final deprotection and the isolation/purification of **1**. Best results are obtained using boron tribromide yielding **1** in 35% yield.

2.2. Anti-integrase activity and antiviral properties

Since there is no significant difference between the activities of L- and D-chicoric acids, we have tested fukiic acid as the racemic form on integrase. *rac*-**1** was tested in IN inhibition assays which have been recently reviewed [14]. The IN inhibition assays were done in two independent laboratories and gave different results mainly due to the presence or the absence of bovine serum albumin (BSA) in the medium assay. Other smaller differences due to the IN purification processes [14,15] and the conditions of the assays did not significantly modify the IC_{50}'s. The IC_{50} values were measured for the overall integration reaction and compared to the inhibitory properties of L-chicoric acid and D-chicoric acid [10]. Results are reported in Table 1. With numerous inhibitors, BSA does not interfere in this assay, but in the present case it seems that the presence of a catechol function may promote an interaction between *rac*-**1** and BSA [16]. Our own experience on numerous polyphenols confirms that the IC_{50} values are 50–100 folds lower with BSA than without BSA.

The antiviral activity on HIV-1-induced CPE in human lymphocyte MT-4 cell culture was determined by the MT-4/MTT-assay [17]. Compound **1** was not found to be active at concentrations lower than the CC_{50}.

Results reported in Table 1 indicated that fukiic acid and chicoric acid have very similar activities against IN but their toxicities are quite different. L- and D-chicoric acids were found to inhibit the overall integration reaction at submicromolar levels via the selective inhibition of the 3′-processing step [10]. This apparent 3′-processing inhibition is due to assembly inhibition [18], but the viral entry was demonstrated to be the primary target for the anti-HIV-1 activity of chicoric acid [10]. Compared to L- and D-chicoric acids, fukiic acid is 5-fold more toxic and its toxicity did not allow us to evaluate its potential activity against the viral replication. It is now well established that selective strand transfer inhibitors also inhibit HIV-1 replication in cell culture [19] (most of the non-selective strand transfer inhibitors were found to present antiviral properties via another target that IN). The lack of cell-based activity of fukiic acid in MT-4 cells could be therefore due to the absence of selectivity towards the second step catalyzed by integrase and/or the absence of another viral target (i.e., entry as in the case of chicoric acid).

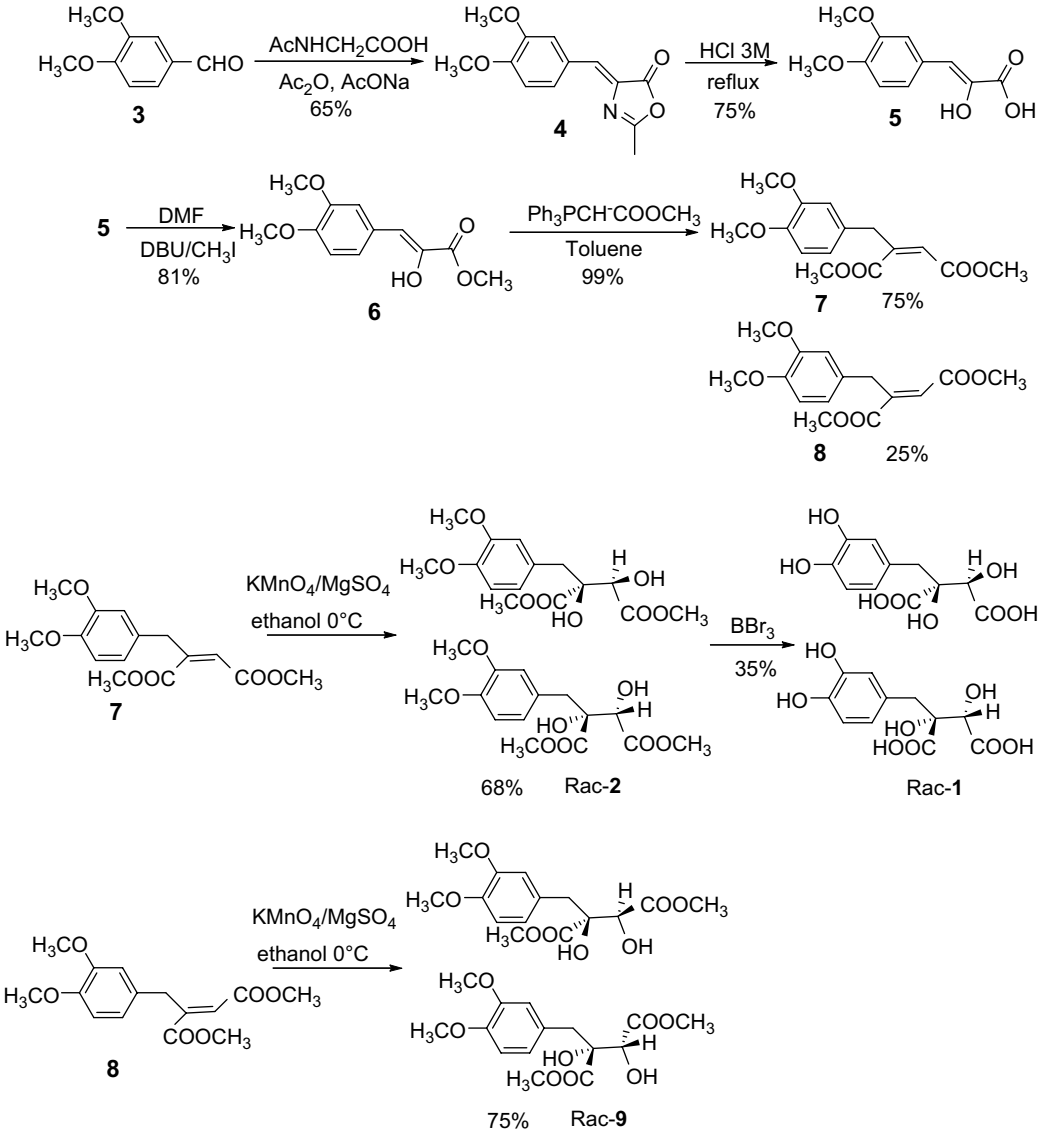
Whereas chicoric acid presented anti-IN and anti-HIV-1 properties, its acid constituents, i.e. caffeic and tartaric acids had no antiviral activities [20]. In fukiic acid, the catechol and tartaric acid moieties are linked by a methylene group. This link yields submicromolar anti-IN activities comparable to those of L- and D-chicoric acids. However, the anti-HIV-1 properties of fukiic acid cannot be measured due to cytotoxicity. The link between catechol and tartaric acid moieties will be modified in order to decrease the cytotoxicity.

In conclusion, we report the first total synthesis of *rac*-fukiic acid, a new submicromolar inhibitor of HIV-1 integrase.

3. Experimental protocols

3.1. Chemistry

All reagents and solvents were purchased from Aldrich-Chimie (Saint-Quentin-Fallavier, France), were of ACS reagent grade and were used as provided. TLC analyses were performed on plastic sheets precoated with silica gel 60F254 (Merck). SiO_2, 200–400 mesh (Merck) was used for column



Scheme 2.

chromatography. NMR spectra were obtained on an AC 200 Bruker spectrometer in the appropriate solvent with TMS as internal reference. Melting points were obtained on a Reichert Thermopan melting point apparatus, equipped with a microscope and are uncorrected. Melting points and spectroscopic data of compounds **7**, **8**, *rac*-**9** and *rac*-**2** are in accordance with the literature [21].

3.1.1. Synthesis of rac-fukiic acid

Compound **2** was treated with boron tribromide 1.0 M in CH_2Cl_2 (5 equiv) for 1 h at room temperature. After hydrolysis at room temperature, the aqueous layer was separated and extracted with ethyl acetate. The washed aqueous layer was concentrated in vacuum and the crude solid was dissolved in hot acetone. The solution was filtered and the solvent was evaporated. The residue was then treated in the same manner with MeOH affording *rac*-fukiic acid **1** in 35% yield as oily syrup. Spectroscopic data are in accordance with the literature [5]. Mp >250 °C; ^{1}H NMR (acetone-d_6) (δ): 2.91 (d, 1H, $^2J = 13.8$ Hz, CH_2); 3.12 (d, 1H, $^2J = 13.8$ Hz, CH_2); 4.53 (s, 1H); 6.52–6.78 (m, 3H, ArH); ^{13}C NMR (δ): 41.5 (t); 75.7 (d); 80.6 (s); 115.5 (d); 118.35 (d); 122.6 (d); 128.1 (s); 144.6 (s); 145.15 (s); 172.9 (s); 174.2 (s).

Table 1
Inhibition of integration and anti-HIV activity, both expressed in μM

Compound	IN inhibition[a]	MT-4 cells	
	IC_{50}	EC_{50}	CC_{50}
L-CA[b]	0.2 ± 0.2	12.7 ± 8.5	115 ± 21
D-CA[b]	0.3 ± 0.02	10.9 ± 6.5	111 ± 25
rac-Fukiic acid	0.25 ± 0.02 17.0 ± 0.02[c]	>22	22 ± 0.02

[a] The IN inhibition was assayed on the overall integration reaction.
[b] Biological data of chicoric acids are from Ref. [10].
[c] BSA was added in the medium.

3.2. Pharmacology

3.2.1. HIV-1 integrase inhibition

3.2.1.1. IN inhibition + BSA. To determine the susceptibility of the HIV-1 integrase enzyme towards different compounds we used an enzyme-linked immunosorbent assay. This assay uses an oligonucleotide substrate of which one oligo (5′-ACTGCTAGAGATTTTCCACACTGACTAAAAGGGTC-3′) is labeled with biotin on the 3′ end and the other oligo is labeled with digoxigenin at the 5′ end. For the overall integration assay the second 5′-digoxigenin labeled oligo is 5′-GACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA**GT**-3′. The integrase enzyme [13] was diluted in 750 mM NaCl, 10 mM Tris, pH 7.6, 10% glycerol and 1 mM β-mercaptoethanol. To perform the reaction, 4 μL diluted integrase (1.6 μM) and 4 μL of oligonucleotides (7 nM) were added in a final volume of 40 μL containing 10 mM $MgCl_2$, 5 mM DTT, 20 mM HEPES, pH 7.5, 5% PEG, 15% DMSO and 0.1 mg/mL BSA. Reaction products were denatured with 30 mM NaOH and detected by an immunosorbent assay on avidin-coated plates [22].

3.2.1.2. IN inhibition − BSA. Oligonucleotides were purchased from Eurogentec and further purified on 18% acrylamide/urea denaturing gel. U5B: GTGTGGAAAATCTCTAGCA; U5A: 5′-ACTGCTAGAGATTTTCCACAC. Wild-type HIV-1 integrase was purified as described previously [14]. The assay was performed in a reaction volume of 20 μL containing 0.025 pmol of labeled U5A/U5B double-stranded DNA substrate and 1 pmol of integrase in buffer [20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 25 mM NaCl, and 1 mM DTT]. Products were separated on an 18% acrylamide/urea denaturing gel and quantified on a phosphoimager using ImageQuant software (Amersham Pharmacia Biotech).

3.2.2. In vitro anti-HIV and drug-susceptibility assays

The inhibitory effect of antiviral drugs on the HIV-induced cytopathic effect (CPE) in human lymphocyte MT-4 cell culture was determined by the MT-4/MTT assay. This assay is based on the reduction of the yellow coloured 3(-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenase of metabolically active cells to a blue formazan derivative, which can be measured spectrophotochemically. The 50% cell culture infective dose ($CCID_{50}$) of the HIV (IIIB) strain was determined by titration of the virus stock using MT-4 cells. For the drug-susceptibility assays, MT-4 cells were infected with 100−300 $CCID_{50}$ of the virus stock in the presence of five-fold serial dilutions of the antiviral drugs. The concentration of various compounds achieving 50% protection against the CPE of the different HIV strains, which is defined as the EC_{50} was determined. In parallel, the 50% cytotoxic concentration (CC_{50}) was determined.

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