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# Synthesis and in vitro antitumour activity of crassalactone D, its stereoisomers and novel cinnamic ester derivatives

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**Abstract**

Naturally occurring styryl lactone, crassalactone D (**1**), unnatural 4-*epi*-crassalactone D (**2**), and the corresponding 7-epimers (**3** and **4**) have been synthesized starting from D-glucose. The key step of the synthesis is a new one-pot sequence that commenced with a *Z*-selective Wittig olefination of suitably functionalized sugar lactols with a stabilized ylide, (methoxycarbonylmethylene)-triphenylphosphorane, in dry methanol, to afford **1** or **3**, in the mixtures with the corresponding 4-epimers (**2** or **4**, respectively). A number of 6-*O*-cinnamoyl derivatives of styryl lactones **1**–**4** have been prepared, bearing electron donating or electron withdrawing functionalities in the C-4 position of cinnamic acid residue. The synthesized products were evaluated for their in vitro antiproliferative activity against selected human tumour cell lines, whereupon very potent cytotoxicities have been recorded in many cases. SAR analysis indicated some important structural features responsible for biological activity, such as stereochemistry at the C-4 and C-7 positions, as well as the nature of a substituent at the C-4 position in the aromatic ring of cinnamoate moiety. Flow cytometry and Western blot analysis data gave insight in the mechanism underlying antiproliferative effects of the synthesized compounds.

# 1. Introduction

Crassalactone D (**1**, Fig. 1) is a naturally occurring styryl-lactone that shows notable cytotoxic effects against certain mammalian tumour cell lines. It was isolated from the tropical plant *Polyalthia crassa* by Tuchinda and co-workers in 2006 [1]. The relative configuration of **1** was established by X-ray diffraction analysis and its absolute stereochemistry was determined by NMR studies of its (*R*)-and (*S*)-MTPA esters. Two asymmetric total syntheses of crassalactone D (**1**) reported in 2009 [2,3] have been followed by a formal synthesis published in 2012 [4]. In the same year, we published a preliminary account describing a new chiral pool approach to the total synthesis of the natural product **1** and several new epimers [5]. The effects of the synthesized compounds on the proliferation of some human malignant cell lines have also been preliminary reported [5]. Herein, we wish to disclose full details of the synthesis and biological evaluation of styryl lactones **1–4**, as well as some new 6-*O*-cinnamoyl derivatives (**1a–d**, **2a–d**, **3a–d** and **4a–d**) along with their effects on the proliferation of certain malignant cell lines. Rationale for the preparation of cinnamic ester derivatives of crassalactone D and epimers arises from the fact that cinnamoates represent a promising class of anti-cancer agents [6]. Such concept of drug design and development is known as 'molecular hybridization'. It is based on the combination of pharmacophoric moieties of different bioactive substances to produce a new hybrid compound with improved affinity and efficacy. Additionally, this strategy can result in compounds presenting modified selectivity profile, different and/or dual modes of action and reduced undesired side effects [7]. Another reason for studying the cinnamic ester derivatives of type **1b–d**, **2b–d**, **3b–d**, **4b–d** is derived from our earlier findings that the introduction of either electron withdrawing or electron donating groups in the C-4 position of the cinnamic acid aromatic ring may increase antitumour activity of the resulting (+)-crassalactone B and C analogues [8,9]. Apoptotic signalling induced by the synthesized compounds was also studied.

<Insert Figure 1>

# 2. Results and discussion

## 2.1. Chemistry

Synthesis of crassalactone D (**1**) and the corresponding 7-epimer **3** is shown in *Scheme 1*. The starting compounds **5** and **7** were prepared from commercially available diacetone-D-glucose in three steps as we reported earlier [10]. Hydrolytic removal of the isopropylidene protective groups in both **5** and **7** afforded the corresponding lactols **6** and **8** in almost quantitative yields. Both

products, and particularly stereoisomer **6**, are rather hygroscopic. They were therefore used in the next synthetic step immediately after their brief isolation. Accordingly, lactol **6** was submitted to the reaction with a stabilized ylide, (methoxycarbonylmethylene)triphenylphosphorane (MCMP) in dry methanol, to afford (+)-crassalactone D (**1**) along with the corresponding 4-epimer **2** in 1:2 respective ratio and in 51% combined yield. This one-pot sequence is comprised of an initial *Z*-selective Wittig olefination [11,12], followed by successive γ-lactonization, β-elimination and the final 5-*endo-trig* spirocyclization [5,10]. In order to increase the yield and/or epimer ratio we carried out the olefination step with (ethoxycarbonylmethylene)triphenylphosphorane (ECMP) [13,14]. However, the isomers **1** and **2** were isolated in the similar total yield (48%) and in the same isomer ratio (**1/2** = 1:2).

<Insert Scheme 1>

In an attempt to generalize this methodology by using a similar substrate, but with the opposite stereochemistry at the C-5 position, we treated lactol **8** under the same Wittig olefination conditions. Gratifyingly, reaction of **8** with MCMP gave unnatural spiro-lactones **3** and **4** in 67% combined yield. Unfortunately, a drop in selectivity was observed, whereupon an equimolar mixture of **3** and **4** was obtained. However, when the Wittig olefination of **8** was performed with ECMP, products **3** and **4** were obtained in respective isomeric ratio of 1:4, in slightly lower total yield (46%). The products **1** and **2**, as well as **3** and **4**, were separated with difficulties. In order to obtain pure products several flash column chromatographic separations were required, followed by preparative TLC. This significantly decreased the yields of all products, and in particular the yields of minor stereoisomers. Fortunately, it was found that the less stable isomer **2** could be converted to the more stable **1**, after treatment of their mixture (2:1 in favour of **2**) with a solution of trifluoroacetic acid in chloroform, to give a 2:1 mixture of **1** and **2** (by ^{1}H NMR analysis). The similar results were obtained when the reaction was carried out in the presence of Lewis acids (for more details, see the Supplementary data). Greater stability of naturally occurring **1** compared to **2**, could be a result of stabilization by anomeric effect and the related stereoelectronic effects [15,16]. In contrast, when pure **4** was treated under similar reaction conditions, a 1:1 mixture of **3** and **4** was obtained indicating a similarity in stability of both stereoisomers.

Both ^{1}H and ^{13}C NMR data and physical properties of compounds **1** and **2** were in agreement with those reported previously [2,3]. Stereochemistry of lactones **3** and **4** were confirmed by single crystal X-ray diffraction analysis (for the crystal structures of **3** and **4**, see the Supplementary data).

Two independent procedures were used for the conversion of spiro-lactones **1–4** to the cinnamic esters **1a–d** and **2a–d** and the results are presented in Table 1.

<Insert Table 1>

In the first experiments, a mixture of stereoisomeric lactones **1** and **2** (in 1:2 respective ratio) was treated with cinnamoyl chloride (entry 1) or with 4-nitrocinnamoyl chloride (entry 2) in the presence of DMAP in dry dichloromethane, to afford mixtures of **1a** and **2a** or **1b** and **2b** in 72 and 85% combined yields, respectively.

Next, the esterification step was carried out by using the Steglich esterification protocol [17]. We were pleased to find that treatment of mixture of **1** and **2** (in 1:2 respective ratio) with 4-methoxycinnamic acid, in the presence of DCC and DMAP in anhydrous dichloromethane, gave the corresponding esters **1c** and **2c** in 32 and 60% respective yields (entry 3). Finally when the mixture of **1** and **2** was treated with 4-fluorocinnamic acid, under the same reaction conditions as those described above, the expected products **1d** and **2d** were obtained in 36 and 50% yields, respectively (entry 4).

The results related to the synthesis of 6-*O*-cinnamoyl derivatives **3a–d** and **4a–d** are presented in Table 2.

<Insert Table 2>

Treatment of **3** with cinnamoyl chloride or with 4-nitrocinnamoyl chloride in the presence of DMAP in dry dichloromethane, gave the corresponding 6-*O*-cinnamoyl derivatives **3a** and **3b** in 68 and 74% respective yields (entries 1 and 2). The same synthetic protocol was then used for the conversion of **4** in **4a** and **4b** in 62 and 89% yields, respectively (entries 5 and 6). Next, the Steglich esterification protocol was used for the synthesis of the corresponding 6-*O*-(4-methoxycinnamoyl) or 6-*O*-(4-fluorocinnamoyl) derivatives (Table 2, entries 3, 4, 7 and 8). Accordingly, a treatment of **3** with 4-methoxycinnamic or with 4-fluorocinnamic acid in anhydrous dichloromethane, in the presence of DCC and DMAP, gave the expected 6-*O*-(4-methoxycinnamoyl) derivative **3c** (entry 3) and 6-*O*-(4-fluorocinnamoyl) derivative **3d** (entry 4) in 86 and 73% yields respectively. Finally, compound **4** reacted with 4-methoxy- or 4-fluorocinnamic acid, under the same reaction conditions, to afford the corresponding 4-substituted cinnamoates **4c** and **4d** in 77 and 88% respective yields (entries 7 and 8).

It should be noted that all C-6 signals of (7*S*)-isomers (**3**, **4**, **3a–d**, **4a–d**) appeared downfield compared to the corresponding signals of (7*R*)-isomers (**1**, **2**, **1a–d**, **2a–d**), presumably due to the steric compressions caused by the OH and Ph groups.

Structure and stereochemistry of esters **1b–d**, **2a** and **4a** were confirmed by single crystal X-ray diffraction analysis (for the crystal structures see the Supplementary data).

*2.2. In vitro antitumour activities and SAR*

Crassalactone D (**1**), its stereoisomers (**2–4**) and 6-*O*-cinnamoyl derivatives (**1a–d**, **2a–d**, **3a–d**, **4a–d**) were evaluated for their *in vitro* antitumour activity against eight human malignant cell lines including myelogenous leukaemia (K562), promyelocytic leukaemia (HL-60), T-cells leukaemia (Jurkat), Burkitt's lymphoma (Raji), oestrogen receptor positive breast adenocarcinoma (MCF-7), oestrogen receptor negative breast adenocarcinoma (MDA-MB-231), cervix carcinoma (HeLa), alveolar basal adenocarcinoma (A549) and a single human normal cell line (MRC-5). Cytotoxic activity was evaluated by using the standard MTT assay [18], after exposure of cells to the tested compounds for 72 h. The commercial antitumour agent doxorubicin (DOX) was used as a reference compound in this assay.

<Insert Table 3>

Crassalactone D (**1**) and stereoisomers **2** and **4** exhibited submicromolar activity against K562 cell line (Table 3). Natural lactone **1** was 2-fold more active than commercial antitumour agent DOX, 5-fold more active with respect to **2** and **4** and 10-fold more potent than **3**. Compound **1** (IC$_{50}$ 0.34 µM) was 6-, 14- and 104-fold more potent compared to **2**, **4** and **3**, respectively in the culture of HeLa cells. Although spiro lactones **1–4** were very potent inhibitors of Jurkat cells growth, all of them were less active than DOX. In the culture of Raji cells the most active was natural product **1** being as active as DOX, but more active than stereoisomers **2–4**. Crassalactone D (**1**) showed the highest potency against MDA-MB231 cells and the least potency against MCF-7 cells, when compared to the remaining styryl lactones **2–4**.

Twelve 6-*O*-cinnamoyl derivatives (**1b–c**, **2b–c**, **3a,d**, and **4a–d**) exhibited activities in the micromolar range toward K562 cells, while thirteen of them (**1b**,**c**, **2a–d**, **3a–c**, and **4a–d**) showed micromolar activity against HL-60 cells. However none of the analogues were more active than DOX in the culture of these cell lines. Cinnamoate **1a** demonstrated a submicromolar cytotoxicity (IC$_{50}$ 0.21 µM) against Jurkat cells, while compounds **3a** and **4a–d** were found to be active in

micromolar range toward the same cell line. In the culture of Raji cells compounds **2a**, **4a** and **4b** exhibited a greater activity than DOX. Cinnamoyl esters **1a–c** were more active than lead **1** toward MCF-7 cells. Compound **2a** was very potent growth inhibitor of MCF-7, MDA-MB 231 and HeLa cells being more active than (4*R*)-crassalactone D (**2**). Derivatives **3a–d** and **4a–d** were more active than lead compounds **3** and **4** against human breast adenocarcinoma cells (ER⁻, MDA-MB 231). These compounds were more potent then leads **3** and **4** and control compound DOX against human adenocarcinoma cells (A549). Moreover, none of the synthesized styryl lactones exhibited any toxicity toward normal MRC-5 cells, in contrast to the commercial antitumour agent doxorubicin that exhibited a strong cytotoxicity in submicromolar range (IC$_{50}$ 0.10 µM).

In an attempt to determine influence of newly introduced cinnamoyl ester groups on antitumour activity, we compared IC$_{50}$ values of cinnamoates **1a–d**, **2a–d**, **3a–d**, **4a–d** with those recorded for leads **1–4** (see the Supplementary data for details). The results have shown that presence of cinnamoyl ester group in molecule **1** increased cytotoxicity of analogue **1a** against MCF-7 and A549 cells, while the introduction of 4-substituted cinnamoyl groups increased cytotoxicity only against MCF-7 cell line. The presence of non-substituted cinnamoyl moiety in the structure of lead **2** increased the potency of resulting analogue **2a** against four malignant cell lines (Raji, MCF-7, MDA-MB231, HeLa), while introduction of electron withdrawing group in the position C-4 of cinnamoyl moiety (NO$_2$ and F) decreased activity of analogues **2b** and **2d** toward the all tumour cell lines under evaluation. However, introduction of electron donating group (OMe) at C-4 of cinnamoyl moiety increased the potency of resulting analogue **2c** only against MCF-7 cell line. Presence of cinnamoyloxy groups at the C-6 position of lead **3** has more favourable effects. Introduction of non-substituted cinnamoyl moiety as well as 4-nitrocinnamoyl residue in the structure of lead **3** increased the potency of resulting analogues **3a** and **3b** against majority of tumour cell lines under evaluation, while introduction of 4-methoxy- and 4-fluorocinnamoyl moieties increased activity of analogues **3c** and **3d** against three investigated cell lines (MDA-MB231, HeLa, A549). Cinnamoates **4a** and **4b** were more active against three cell lines (MDA-MB231, A549, Raji), while analogues **4c** and **4d** were more active against two cell lines (MDA-MB231, A549), compared to parent compound **4**. These results implied that introduction of cinnamoyloxy groups at the C-6 positions of leads **3** and **4** may increase antitumour activities of 6-*O*-cinnamoyl derivatives.

Next we considered the influence of electron withdrawing (NO$_2$, F) and electron donating (OMe) groups attached in the C-4 position of cinnamate aromatic ring. Non-substituted cinnamoyl derivatives **1a**, **2a**, **3a** and **4a** were used as controls in this SAR analysis. Insertion of electron

donating methoxy substituent in the C-4 position of cinnamic acid moiety in derivatives **1a**, **2a** and **3a** influenced increase in activity toward two (**1c**), or three (**2c** and **3c**) cell lines. The most significant influence methoxy group was recorded in **4c** that exhibited higher potencies against five cell lines with respect to non-substituted cinnamate **4a**. The presence of electron withdrawing fluoro group at the C-4 position of cinnamoyl ester group of **1a** lead to increase of potency only against K562 cells (compound **1d**). The presence of nitro group however, influenced increase of activity against both K562 and HL-60 cells (**1b** and **2b**), while insertion of fluoro group in **2a** lead to favourable antitumour properties compared to nitro group (four *vs.* two cell lines). Presence of both fluoro and nitro group in the C-4 position of cinnamic acid moiety of **3a** exhibited the same effects, that is increase of cytotoxicity against HeLa and MDA-MB231 cells. Analogues with fluoro (**4d**) and nitro (**4b**) groups showed higher potency toward three i.e. six tumour cell lines, respectively compared to control **4a**. These results indicated that introduction of either electron withdrawing (NO2 and F) or electron donating (OMe) groups in the C-4 position of cinnamoyloxy moiety decreases antitumour potencies against majority tumour cell lines under evaluation.

In the next phase of SAR analysis we compared $IC_{50}$ values of derivatives with electron donating (**1c**, **2c**, **3c**, **4c**) and electron withdrawing (**1b,d**, **2b,d**, **3b,d**, **4b,d**,) groups in the position C-4 of cinnamate aromatic ring. The results showed that compounds bearing a methoxy group at the position C-4 in cinnamoyloxy moiety were more active then 4-fluoro and 4-nitro derivatives, with one exception (**2c** *vs.* **2d**, three *vs.* five cell lines).

Finally, we considered influence of absolute stereochemistry at C-4 on antitumour activity. The data in Table 3 indicate that (4*R*)-stereoisomers showed higher potency toward majority of investigated cell lines compared to (4*S*)-isomers with one exception. Namely, 4-nitrocinnamoyl-crasslactone D (**1b**) exhibited higher cytotoxicity against five of eight cell lines under investigation compared to (4*R*)-analogue (**2b**).

Our previous studies [8–10] indicated that the styryl lactones having the (7*S*)-stereochemistry represent more potent cytotoxic agents with respect to the corresponding (7*R*)-epimers. The results obtained from comparison of the $IC_{50}$ values of (7*R*)- and (7*S*)-crassalactone D derivatives, which showed that (7*S*)-stereoisomers were are more active then (7*R*)-analogues toward the majority of investigated cell lines, agrees well with our previous findings [8–10].

*2.3. Detection of apoptosis and apoptotic pathways*

Apoptosis is a cell suicide program that plays a critical role in development and tissue homeostasis. The ability of cancer cells to evade this programmed cell death is one of the major characteristics that enables their uncontrolled growth [19]. The efficiency of chemotherapy in killing such cells depends on the successful induction of apoptosis, since defects in apoptosis signalling represents one of the causes of drug resistance [20].

Recent report disclosed by Choo et al [21] showed that some of natural styryl-lactones isolated from *Goniothalamus* genus induced apoptosis in various tumour cell lines. This finding prompted us to examine apoptotic signalling induced by (+)-crassalactone D (**1**), its epimers **2–4** and cinnamoyl derivatives **1a–d**, **2a–d**, **3a–d**, **4a–d**.

Influence of the synthesized compounds on cell distribution in cell cycle phases was investigated first. After treatment of K562 cells with the synthesized compounds at their $IC_{50}$ concentrations, the cells were permeabilised and treated with a fluorescent dye (propidium iodide, PI) that stains DNA quantitatively. The fluorescence intensity of the stained cells at certain wavelengths is in correlation with the amount of DNA they contain, hence staining of apoptotic cells resulted in broad hypodiploid picks after flow cytometry analysis.

As data in Table 4A reveal crassalactone D (**1**) slightly increased, while its epimers **2–4** decreased percentage of K562 cells in sub G1 phase compared with untreated control. All cinnamoyl derivatives **1a–d**, **2a–d**, **3a–d** and **4a–d** increased the percent of cells in sub G1 phase compared both with leads **1–4** and control. Significant accumulation of cells in the sub G1 phase was detected after treatment of cells with compounds **1a** (19-fold), **2a** (38-fold), **3b** (14-fold), **3d** (10-fold), **4a** (8-fold) and **4b** (27-fold) along with the decrease of cells in G0G1 and S phase of cell cycle. Cinnamoates **1c**, **1d, 3a**, **3c** and **4d** significantly increased the cell percentage in G2/M phase of cell cycle.

The observed apoptosis-inducing effect of investigated compounds (Table 4A) was also confirmed by flow cytometric evaluation of Annexin V binding, which measures phosphatidyl serine on the external leaflet of the plasma membrane, an event characteristic of early apoptosis.

Double staining (PI and Annexin V) of K562 cells treated with the synthesized compounds followed by fluorescence activated cell storing (FACS) enabled differentiation of normal, living cells (low Annexin and low PI staining), apoptotic cells (high Annexin and low PI staining) and necrotic cells (low Annexin and high PI staining). Results are presented in Table 4B as the percentage of specific apoptosis and necrosis [22].

<Insert Table 4>

Although natural product **1** just slightly increases percentage of K562 cells in sub G1 phase with respect to control, it induced apoptosis in 22.6% of cells. Similarly, 6-*O*-cinnamoyl derivative **3a** increased only 3.8-fold percentage of K562 cells in sub G1 phase, but induced apoptosis in 81.79% cells. Very high percentage of specific apoptosis after treatment of K562 cells with cinnamoates **1a** (83.35%), **2a** (86.01%), **3b** (81.97%), **3d** (85.69%) and **4b** (70.03%) is in agreement with detection of apoptotic DNA fragmentation (increased sub G1 cell fraction), an event which follows the activation of executioner caspases. All tested compounds induced low percentage of specific necrosis in K562 cells (0.64–4.06%), except compound **4a** (28.29%).

Next, we investigated how the synthesized compounds modulate expression of some apoptosis proteins (Bcl-2, Bax, caspase 3 and PARP). Diverse cytotoxic stimuli, including oncogenic stress and chemotherapeutic agents could engage the mitochondrial apoptosis pathway, which is regulated by Bcl-2 family members. These stimuli activate BH3-only family members (initiators), which inhibit the pro-survival Bcl-2-like proteins (guardians), thereby enabling activation of the pro-apoptotic effectors Bax and Bak, which then disrupt the mitochondrial outer membrane. Released cytochrome *c* promotes caspase activation [23].

Caspase 3 is the key executioner caspase, and it exists as an inactive zymogen that is activated by upstream signals. Caspase 3 could be activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [24]. The conversion of procaspase 3 to caspase 3 results in the generation of the active 'executioner' caspase that subsequently catalyzes the hydrolysis of many protein substrates [24] and other downstream targets including PARP which primary function is to detect and repair DNA damage. Cleavage of PARP by caspases is considered to be a hallmark of apoptosis [25].

<Insert Figure 2>

Western blot analysis revealed that five (**1**, **4a–d**) of 20 evaluated compounds decreased expression of both anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein in K562 cells compared with control (Fig. 2). Compounds **1**, **4a–d** increased expression of activated caspase 3 or 85 kDa fragment of cleaved PARP or both which allowed us to hypothesize that these compounds induce apoptosis in caspase-dependent way.

Lactones **1a**, **2a**, **2d**, **3**, **3a**, **3b**, **3d**, **4** decreased expression of anti-apoptotic Bcl-2 protein and increased of pro-apoptotic Bax protein while **1b–d**, **2**, **2b**, **2c** and **3c** increased expression of both Bcl-2 and Bax. Majority of these compounds induced over-expression of activated caspase 3 subunit or 85 kDa fragment of cleaved PARP or both (with the exception of **1b** and **3a** which decreased both). Hence, these results have suggested that the triggered caspase-dependent apoptotic cell death was influenced with the Bcl-2 protein family.

## 3. Conclusions

A novel synthesis of crassalactone D (**1**) and its epimers **2–4** has been achieved starting from the commercially available chiral template, diacetone-D-glucose. Synthesis of natural **1** has been achieved employing a new one-pot process that started with a *Z*-selective Wittig reaction of lactol **6** with MCMP or ECMP as a key step, followed by acid-promoted equilibration of dominant isomer **2**. Spiro-lactones **3** and **4** were obtained in a similar manner from monoacetonide **7**. Compounds **1–4** were treated with cinnamoyl chloride, *p*-nitrocinnamoyl chloride, *p*-methoxycinnamic acid and *p*-fluorocinnamic acid to afford the corresponding ester derivatives **1a–d**, **2a–d**, **3a–d** and **4a–d**.

Evaluation of antitumour activity *in vitro* showed that all of the synthesized compounds, with the exception of **2b**, showed higher potency then natural product **1** against at least one tumour cell line. Eleven of twenty synthesized compounds were more active then DOX against at least one cell line, while none of them was active against the normal MRC-5 cells. The preliminary SAR study reveals that an electron-donating group attached in the para position of cinnamoate aromatic ring is beneficial for antiproliferative activity, as well as (4*R*) i.e. (7*S*) stereochemistry. The flow-cytometry and Western blot analysis showed that apoptotic cell death induced by majority of the synthesized compounds was caspase-dependent.

## 4. Experimental section

### 4.1. Chemistry

#### 4.1.1. General experimental procedures

Melting points were determined on a Hot Stage Microscope Nagema PHMK 05 and were not corrected. Optical rotations were measured on an Autopol IV (Rudolph Research) polarimeter at room temperature. NMR spectra were recorded on a Bruker AC 250 E or a Bruker Avance III 400 MHz instrument and chemical shifts are expressed in ppm downfield from TMS. IR spectra were recorded with an FTIR Nexus 670 spectrophotometer (Thermo-Nicolet). High resolution mass

spectra (ESI) were acquired on a Agilent Technologies 1200 series instrument equipped with Zorbax Eclipse Plus C18 (100 mm × 2.1 mm i.d. 1.8 μm) column and DAD detector (190–450 nm) in combination with a 6210 time-of-flight LC/MS instrument (ESI) in the positive ion mode. Flash column chromatography was performed using Kieselgel 60 (0.040–0.063, E. Merck). Preparative TLC was performed on hand-made plates, 20 × 20 cm size with ~1 mm layer thickness. Kieselgel 60 G (E. Merck) with fluorescent indicator $F_{254}$ as additive was used as stationary phase. The corresponding bands were scraped and eluted with EtOAc. All organic extracts were dried with anhydrous $Na_2SO_4$. Organic solutions were concentrated in a rotary evaporator under diminished pressure at a bath temperature below 35 °C.

### 4.1.2. (+)-Crassalactone D (1) and (4R)-(+)-crassalactone D (2)

*Procedure A*: A solution of **5** (0.443 g, 1.51 mmol) in 90% TFA (6.1 mL) was stirred for 30 minutes at 0 °C and then for additional 1 h at room temperature. The mixture was concentrated by co-distillation with toluene and the residue was purified by flash column chromatography (24:1 $CH_2Cl_2$/MeOH) to afford pure hygroscopic syrup **6** (0.380 g, 100%). To a cooled (0 °C) solution of lactol **6** (0.380 g, 1.51 mmol) in dry MeOH (38 mL) was added MCMP (0.753 g, 2.32 mmol). After 30 min cooling bath was removed and the mixture was stirred at room temperature for 3 h. Another portion of MCMP (0.540 g, 1.51 mmol) was added following previous procedure but stirring was continued at room temperature for another 18 h. The mixture was concentrated. Multiple purification by flesh column chromatography (4:1 $Et_2O$/light petroleum, 17:3 $CH_2Cl_2$/EtOAc) afforded pure mixture of **1** and **2** (0.179 g, 51%), in the respective ratio of 1:2 ($^1$H NMR).

*Procedure B*: To a solution of **1** and **2** (1:2 mixture, 0.174 g, 0.75 mmol) in dry $CH_2Cl_2$ (3.6 mL) was added TFA (0.56 mL, 7.59 mmol). Reaction mixture was stirred for 16 h at room temperature and then concentrated by co-distillation with toluene. The mixture of **1** and **2** (99%) was obtained in the respective ratio of 2:1 ($^1$H NMR). Separation by preparative TLC (15:1 → 1:3 hexane/EtOAc, two successive developments) gave pure **1** (0.059 g, 17%) and **2** (0.031 g, 9%).

### 4.1.2.1. (+)-Crassalactone D (1)

Colourless needles, mp 140–142 °C, (EtOAc/hexane), $[\alpha]_D$=+20.5 (*c* 0.2, EtOH),[*] $R_f$=0.63 (5:2 EtOAc/hexane), Ref. [3] mp 138–140 °C, $[\alpha]_D$=+13.6 (*c* 0.2, EtOH). Both $^1$H and $^{13}$C NMR data of

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[*] Our optical rotation value is slightly greater than the data for $[\alpha]_D$ reported in ref 1 {$[\alpha]_D$ =+7 (*c* 0.2, EtOH)}, but were in reasonable agreement with the reported data for synthetic (+)-crassalactone D reported in ref 3.

compound **1** are consistent with the naturally occurring (+)-crassalactone D [1] and its physical properties are in agreement with those reported in the literature [3].

*4.1.2.2. (4R)-(+)-crassalactone D (2)*

Colourless needles, mp 137–142 °C, (EtOAc/hexane), [α]$_D$=+28.0 (*c* 0.5, EtOH), R$_f$=0.61 (5:2 EtOAc/hexane). ^{1}H and ^{13}C NMR data for **4** matched those previously recorded by us [5].

*4.1.3. (7S)-(+)-crassalactone D (3) and (4R,7S)-(+)-crassalactone D (4)*

*Procedure A*. Solution of **7** (0.117 g, 0.40 mmol) in 90% TFA (3.4 mL) was stirred for 30 minutes at 0 °C and then for additional 1.5 h at room temperature. The mixture was concentrated by co-distillation with toluene and the residue was purified by flash column chromatography (47:3→ 23:2 CH$_2$Cl$_2$/MeOH) to afford pure hygroscopic syrup **8** (0.097 g, 96%). To a cooled (0 °C) solution of lactol **8** (0.097 g, 0.38 mmol) in dry MeOH (10 mL) was added MCMP (0.193 g, 0.58 mmol). After 30 min cooling bath was removed and the mixture was stirred at room temperature for 3 h. Another portion of MCMP (0.129 g, 0.38 mmol) was added following previous procedure but stirring was continued at room temperature for another 44 h. The mixture was concentrated. Multiple purification by flesh column chromatography (9:1 Et$_2$O/light petroleum, 17:3 CH$_2$Cl$_2$/EtOAc) afforded pure **4** (0.034 g, 38%) and **3** (0.026g, 29%).

*Procedure B*. Solution of **7** (0.070 g, 0.24 mmol) in 90% TFA (2 mL) was stirred for 30 minutes at 0 °C and then for additional 1.5 h at room temperature. The mixture was concentrated by co-distillation with toluene and the residue was purified by flash column chromatography (47:3→ 23:2 CH$_2$Cl$_2$/MeOH) to afford pure hygroscopic syrup **8** (0.059 g, 96%). To a cooled (0 °C) solution of lactol **8** (0.059 g, 0.23 mmol) in dry MeOH (6 mL) was added ECMP (0.122 g, 0.35 mmol). After 30 min cooling bath was removed and the mixture was stirred at room temperature for 3 h. Another portion of ECMP (0.081 g, 0.23 mmol) was added following previous procedure but stirring was continued at room temperature for another 22 h. The mixture was concentrated. Multiple purification by flesh column chromatography (9:1 Et$_2$O/light petroleum, 17:3 CH$_2$Cl$_2$/EtOAc) afforded pure **4** (0.020 g, 37%) and **3** (0.0054g, 10%).

*Procedure C*: A solution of **4** (0.035 g, 0.15 mmol) in CHCl$_3$ (0.8 mL) and TFA (0.11 mL, 1.51 mmol) was stirred for 23 h at room temperature. The mixture was concentrated by co-distillation with toluene and residue purified by flesh column chromatography (7:3 Et$_2$O/light petroleum). An inseparable mixture of **3** and **4** in the respective 1:2 ratio (^{1}H NMR) was obtained in an almost

quantitative yield. Repeated flash column chromatography gave pure **3** (0.014 g, 41%) and **4** (0.018 g, 51%).

### 4.1.3.1. (7S)-(+)-crassalactone D (3)

Colourless needles, mp 170–175 °C (CH$_2$Cl$_2$/hexane), [α]$_D$=+32.0 (*c* 0.2, EtOH), R$_f$=0.24 (Et$_2$O). $^1$H and $^{13}$C NMR data for **4** matched those previously recorded by us [5].

### 4.1.3.2. (4R,7S)-(+)-crassalactone D (4)

Colourless needles, mp 139–141 °C (EtOAc/hexane), [α]$_D$=+73.0 (*c* 0.2, EtOH), R$_f$=0.54 (Et$_2$O). $^1$H and $^{13}$C NMR data for **4** matched those previously recorded by us [5].

### 4.1.4. General procedure for the preparation of cinnamoates 1a–4a

To a cooled (0 °C) and stirred solution of cinnamoyl-chloride (1.28 equiv) and DMAP (1.56 equiv) in dry CH$_2$Cl$_2$ (0.12–0.16 M) was added a solution of **1–4** (1 equiv)$^†$ in dry CH$_2$Cl$_2$ (0.04 M). After stirring at 0 °C for 2 h additional portions of cinnamoyl-chloride (0.064 equiv) and DMAP (0.77 equiv) were added. The mixture was stirred at 0 °C for 1 h and then at room temperature for the next 20 h (for **1** and **2**), 45 minutes (for **3**) or 22.5 h (for **4**). The mixture was poured into 5–10% aq HCl and extracted with CH$_2$Cl$_2$. The combined extracts were washed with 10% aq NaCl (to pH 7), dried and evaporated. The residue was purified by preparative TLC (1:1 Et$_2$O/light petroleum, two successive developments for **1a**, **2a** and **4a**; 4:1 Et$_2$O/light petroleum for **3a**).

### 4.1.4.1. 6-O-cinnamoyl-(+)-crassalactone D (1a)

Yield 34%. White powder, mp 123–126 °C (CH$_2$Cl$_2$/hexane), [α]$_D$=+118.0 (*c* 0.1, CHCl$_3$), R$_f$=0.31 (1:1 Et$_2$O/light petroleum). IR (film): ν$_{max}$ 1775 and 1713 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.47 (dd, 1 H, $J_{5a,5b}$=14.8, $J_{5a,6}$=1.3 Hz, H-5a), 2.73 (dd, 1 H, $J_{5a,5b}$=14.8, $J_{5b,6}$=6.9 Hz, H-5b), 5.45 (m, 1 H, $J_{5a,6}$=1.3, $J_{6,7}$=2.6, $J_{5b,6}$=6.9 Hz, H-6), 5.57 (d, 1 H, $J_{6,7}$=2.6 Hz, H-7), 6.30 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.57 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2′), 7.31 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.34–7.65 (m, 10 H, 2×Ph), 7.82 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-3'). NOE contact: H-3 and *ortho* H-atoms from Ph group at C-7. $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 40.2 (C-5), 78.9 (C-6), 88.0 (C-7), 113.7 (C-4), 117.2 (C-2′), 125.0 (C-2), 125.4, 128.3, 128.7, 128.9, 129.3, 130.6, 134.0, 137.8 (2×Ph), 146.2 (C-3′), 151.1 (C-3), 166.6 (C-1′), 169.6 (C-1). HRMS (ESI): *m/z* 363.1228 (M$^+$+H), calcd for C$_{22}$H$_{19}$O$_5$: 363.1227;

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$^†$ Compounds **1** and **2** were used in the reaction as a mixture of 1:2, respectively.

$m/z$ 380.1491 ($M^++NH_4$), calcd for $C_{22}H_{22}NO_5$: 380.1492; $m/z$ 385.1045 ($M^++Na$), calcd for $C_{22}H_{18}NaO_5$: 385.1046.

### 4.1.4.2. (4R)-6-O-cinnamoyl-(+)-crassalactone D (2a)

Yield 38%. Colourless needles, mp 112–115 °C ($CH_2Cl_2$/hexane), $[\alpha]_D$=+39.0 ($c$ 0.1, $CHCl_3$), $R_f$=0.46 (1:1 $Et_2O$/light petroleum). IR (film): $\nu_{max}$ 1777 and 1716 (C=O). $^1$H NMR (250 MHz, $CDCl_3$): δ 2.48 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5a,6}$=4.3 Hz, H-5a), 2.90 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5b,6}$=5.6 Hz, H-5b), 5.42 (d, 1 H, $J_{6,7}$=3.2 Hz, H-7), 5.54 (m, 1 H, H-6), 6.21 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.51 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2′), 7.35 (d, 1 H, $J_{2,3}$=5.6 Hz, H-3), 7.38–7.65 (m, 10 H, 2×Ph), 7.78 (d, 1 H, $J_{2',3}$=16.0 Hz, H-3′). NOE contact: H-7 and H-3. $^{13}$C NMR (62.5 MHz, $CDCl_3$): δ 39.1 (C-5), 79.8 (C-6), 87.6 (C-7), 114.2 (C-4), 116.8 (C-2'), 122.9 (C-2), 125.8, 128.2, 128.3, 128.7, 129.0, 130.8, 133.9, 138.0 (2×Ph), 146.4 (C-3'), 152.7 (C-3), 166.0 (C-1′), 170.2 (C-1). HRMS (ESI): $m/z$ 363.1232 ($M^++H$), calcd for $C_{22}H_{19}O_5$: 363.1227; $m/z$ 380.1493 ($M^++NH_4$), calcd for $C_{22}H_{22}NO_5$: 380.1492; $m/z$ 385.1048($M^++Na$), calcd for $C_{22}H_{18}NaO_5$: 385.1046.

### 4.1.4.3. (7S)-6-O-cinnamoyl-(+)-crassalactone D (3a)

Yield 68%. Colourless crystals, mp 168–172 °C ($CH_2Cl_2$/hexane), $[\alpha]_D$=+36.5 ($c$ 0.2, $CHCl_3$), $R_f$=0.42 (4:1 $Et_2O$/light petroleum). IR (film): $\nu_{max}$ 1776 and 1708 (C=O). $^1$H NMR (250 MHz, $CDCl_3$): δ 2.63 (d, 1 H, $J_{5a,5b}$=14.6, H-5a), 2.69 (dd, 1 H, $J_{5a,5b}$=14.7, $J_{5b,6}$=5.3 Hz, H-5b), 5.52 (d, 1 H, $J_{6,7}$=4.5 Hz, H-7), 5.81 (t, 1 H, J=4.8 Hz, H-6), 6.20 (d, 1 H, $J_{2',3}$=16.0 Hz, H-2′), 6.26 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 7.23 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.25–7.55 (m, 11 H, 2×Ph and H-3′). $^{13}$C NMR (62.5 MHz, $CDCl_3$): δ 42.3 (C-5), 73.0 (C-6), 87.4 (C-7), 113.5 (C-4), 117.0 (C-2'), 124.5 (C-2), 127.4, 128.0, 128.2, 128.3, 128.8, 130.4, 134.1, 134.8 (2×Ph), 145.6 (C-3'), 151.8 (C-3), 165.6 (C-1′), 169.9 (C-1). HRMS (ESI): m/z 363.1223 ($M^++H$), calcd for $C_{22}H_{19}O_5$: 363.1227; m/z 380.1486 ($M^++NH_4$), calcd for $C_{22}H_{22}NO_5$: 380.1492; m/z 385.1038 ($M^++Na$), calcd for $C_{22}H_{18}NaO_5$: 385.1046.

### 4.1.4.4. (4R,7S)-6-O-cinnamoyl-(+)-crassalactone D (4a)

Yield 62%. Colourless needles, mp 163–166 °C ($CH_2Cl_2$/hexane), $[\alpha]_D$=+51.0 ($c$ 0.2, $CHCl_3$), $R_f$=0.58 (1:1 $Et_2O$/light petroleum). IR (film): $\nu_{max}$ 1775 and 1713 (C=O). $^1$H NMR (250 MHz, $CDCl_3$): δ 2.63 (dd, 1 H, $J_{5a,5b}$=15.1, $J_{5a,6}$=2.6 Hz, H-5a), 3.00 (dd, 1 H, $J_{5a,5b}$=15.1, $J_{5b,6}$=6.4 Hz, H-5b), 5.59 (d, 1 H, $J_{6,7}$=4.4 Hz, H-7), 5.54 (m, 1 H, H-6), 6.14 (d, 1 H, $J_{2',3}$=16.0 Hz, H-2′), 6.25 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 7.26–7.65 (m, 12 H, 2×Ph, H-3 and H-3′). $^{13}$C NMR (62.5 MHz, $CDCl_3$): δ

42.1 (C-5), 74.4 (C-6), 84.9 (C-7), 112.6 (C-4), 116.8 (C-2′), 124.0 (C-2), 126.9, 128.0, 128.1, 128.2, 128.8, 130.5, 133.9, 134.2 (2×Ph), 145.6 (C-3′), 151.6 (C-3), 165.4 (C-1′), 169.5 (C-1). HRMS (ESI): $m/z$ 363.1220 (M$^+$+H), calcd for $C_{22}H_{19}O_5$: 363.1227; $m/z$ 380.1486 (M$^+$+NH$_4$), calcd for $C_{22}H_{22}NO_5$: 380.1492; $m/z$ 385.1036 (M$^+$+Na), calcd for $C_{22}H_{18}NaO_5$: 385.1046.

### 4.1.5. General procedure for the preparation of 4-nitrocinnamoates *1b–4b*

To a cooled (0 °C) solution of **1–4** (1 equiv)$^†$ in dry $CH_2Cl_2$ (0.06–0.07 M) were added 4-nitrocinnamoyl chloride (1.3–1.7 equiv) and DMAP (1.6–2.1 equiv). After stirring at 0 °C for 1.5–2 h additional portions of 4-nitrocinnamoyl chloride (0.6–0.8 equiv) and DMAP (0.8–1 equiv) were added. The mixture was stirred at 0 °C for another 1–2 h. The mixture was poured into 5–10% aq NaCl and extracted with $CH_2Cl_2$. The combined extracts were dried and evaporated. The residue was purified by preparative TLC (3:2 light petroleum/EtOAc, three successive developments for **1b** and **2b**; 7:3 light petroleum/EtOAc, two successive developments for **4b**) or by flash column chromatography (3:2 light petroleum/EtOAc for **3b**).

### 4.1.5.1. 6-O-(4-nitrocinnamoyl)-(+)-crassalactone D (*1b*)

Yield 29%. Colourless plates, mp 189–191 °C (EtOH), [α]$_D$=+52.0 (*c* 0.1, CHCl$_3$), R$_f$=0.43 (3:2 light petroleum/EtOAc). IR (film): ν$_{max}$ 1774 and 1717 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.47 (bd, 1 H, $J_{5a,5b}$=14.7, H-5a), 2.73 (dd, 1 H, $J_{5a,5b}$=14.7, $J_{5b,6}$=6.7 Hz, H-5b), 5.47 (m, 1 H, H-6), 5.57 (d, 1 H, $J_{6,7}$=2.0 Hz, H-7), 6.32 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.69 (d, 1 H, $J_{2′,3′}$=15.9 Hz, H-2′), 7.34 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.34–7.49 (m, 5 H, Ph), 7.74 and 8.29 (2×d, 4 H, $J$=8.6 Hz, *p*-NO$_2$Ph), 7.84 (d, 1 H, $J_{2′,3′}$=16.0 Hz, H-3′). $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 40.1 (C-5), 79.5 (C-6), 88.1 (C-7), 113.7 (C-4), 121.6 (C-2′), 125.1 (C-2), 124.2, 128.4, 128.5, 128.8, 128.9, 137.7, 140.1, 148.8 (2×Ph), 143.2 (C-3′), 151.0 (C-3), 165.7 (C-1′), 169.6 (C-1). HRMS (ESI): $m/z$ 408.1069 (M$^+$+H), calcd for $C_{22}H_{18}NO_7$: 408.1078; $m/z$ 425.1333 (M$^+$+NH$_4$), calcd for $C_{22}H_{21}N_2O_7$: 425.1343; $m/z$ 430.0893 (M$^+$+Na), calcd for $C_{22}H_{17}NaO_7$: 430.0897.

### 4.1.5.2. (4R)-6-O-(4-nitrocinnamoyl)-(+)-crassalactone D (*2b*)

Yield 56%. White powder, mp 162–165 °C (EtOH), [α]$_D$=+24.0 (*c* 0.1, CHCl$_3$), R$_f$=0.51 (3:2 light petroleum/EtOAc). IR (film): ν$_{max}$ 1774 and 1720 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.49 (dd, 1 H, $J_{5a,5b}$=14.2, $J_{5a,6}$=4.7 Hz, H-5a), 2.92 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5b,6}$=5.8 Hz, H-5b), 5.40 (d, 1 H, $J_{6,7}$=3.5 Hz, H-7), 5.56 (m, 1 H, H-6), 6.23 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.63 (d, 1 H, $J_{2′,3′}$=16.0 Hz, H-2′), 7.31 (d, 1 H, $J_{2,3}$=5.4 Hz, H-3), 7.38–7.63 (m, 5 H, Ph), 7.72 and 8.29 (2×d, 4 H, $J$=8.7 Hz, *p*-

NO$_2$**Ph**), 7.80 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-3'). NOE contact: H-7 and H-3. $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 39.2 (C-5), 80.2 (C-6), 87.5 (C-7), 114.0 (C-4), 121.1 (C-2'), 123.2 (C-2), 124.2, 125.9, 128.5, 128.8, 128.83, 137.7, 139.9, 148.8 (2×Ph), 143.4 (C-3'), 152.4 (C-3), 165.2 (C-1'), 169.4 (C-1). HRMS (ESI): *m/z* 408.1070 (M$^+$+H), calcd for C$_{22}$H$_{18}$NO$_7$: 408.1078; *m/z* 425.1338 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$N$_2$O$_7$: 425.1343.

### 4.1.5.3. (7S)-6-O-(4-nitrocinnamoyl)-(+)-crassalactone D (3b)

Yield 74%. White powder, mp 105–125 °C (Et$_2$O/hexane), [α]$_D$=+21.0 (*c* 0.2, CHCl$_3$), R$_f$=0.37 (1:1 light petroleum/EtOAc). IR (film): ν$_{max}$ 1773 and 1721 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.64 (d, 1 H, $J_{5a,5b}$=14.6 Hz, H-5a), 2.82 (dd, 1 H, $J_{5a,5b}$=14.7, $J_{5b,6}$=5.3 Hz, H-5b), 5.55 (d, 1 H, $J_{6,7}$=4.4 Hz, H-7), 5.83 (t, 1 H, *J*=4.8 Hz, H-6), 6.27 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.31 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2'), 7.23 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.15–8.30 (m, 10 H, 2×Ph and H-3'). $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 42.3 (C-5), 73.5 (C-6), 87.4 (C-7), 113.5 (C-4), 121.3 (C-2'), 124.1 (C-2), 124.6, 127.1, 128.0, 128.4, 128.8, 134.8, 140.2, 148.5 (2×Ph), 142.5 (C-3'), 151.7 (C-3), 164.6 (C-1'), 169.9 (C-1). HRMS (ESI): *m/z* 425.1340 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$NO$_7$: 425.1343.

### 4.1.5.4. (4R,7S)-6-O-(4-nitrocinnamoyl)-(+)-crassalactone D (4b)

Yield 89%. White powder, mp 85–90 °C (EtOH), [α]$_D$=+18.0 (*c* 0.1, CHCl$_3$), R$_f$=0.46 (13:7 light petroleum/EtOAc). IR (film): ν$_{max}$ 1775 and 1720 (C=O). $^1$H NMR (400 MHz, CDCl$_3$): δ 2.63 (dd, 1 H, $J_{5a,5b}$=15.2, $J_{5a,6}$=2.8 Hz, H-5a), 3.04 (dd, 1 H, $J_{5a,5b}$=15.2, $J_{5b,6}$=6.5 Hz, H-5b), 5.62 (d, 1 H, $J_{6,7}$=4.5 Hz, H-7), 5.89 (ddd, 1 H, $J_{5a,6}$=2.8, $J_{6,7}$=4.5, $J_{5b,6}$=6.5 Hz, H-6), 6.26 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2'), 6.28 (d, $J_{2,3}$=5.5 Hz, H-2), 7.21–8.30 (m, 11 H, 2×Ph, H-3 and H-3'). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 42.1 (C-5), 74.9 (C-6), 84.9 (C-7), 112.5 (C-4), 121.0 (C-2'), 124.2 (C-2), 124.3, 127.0, 128.2, 128.4, 128.7, 134.2, 140.0, 148.6 (2×Ph), 142.6 (C-3'), 151.4 (C-3), 164.6 (C-1'), 169.4 (C-1). HRMS (ESI): *m/z* 425.1341 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$NO$_7$: 425.1343.

### 4.1.6. General procedure for the preparation of 4-methoxycinnamoates 1c–4c

To a solution of **1–4** (1 equiv)$^†$ in dry CH$_2$Cl$_2$ (0.03–0.04 M) were added 4-methoxycinnamic acid (2.1 equiv), DMAP (4.2 equiv) and DCC (2.5 equiv). The mixture was stirred at room temperature for 3–5 h, then poured into water and extracted with CH$_2$Cl$_2$. The combined extracts were dried and evaporated. The residue was purified by flash column chromatography (7:3 light petroleum/EtOAc for **1c** and **2c**; 3:2 light petroleum/EtOAc for **3c**; 3:2 light petroleum/EtOAc for **4c**) and then by

preparative TLC (7:3 light petroleum/EtOAc for **1c** and **2c**, two successive developments; 3:2 light petroleum/EtOAc for **3c**; 4:1 light petroleum/EtOAc three successive developments for **4c**).

### 4.1.6.1. 6-O-(4-methoxycinnamoyl)-(+)-crassalactone D (1c)

Yield 32%. Colourless needles, mp 153–156 °C (CH$_2$Cl$_2$/hexane), $[\alpha]_D$=+77.0 (*c* 0.1, CHCl$_3$), R$_f$=0.26 (7:3 light petroleum/EtOAc). IR (film): $\nu_{max}$ 1773 and 1709 (C=O). ^{1}H NMR (250 MHz, CDCl$_3$): δ 2.46 (dd, 1 H, $J_{5a,5b}$=14.8, $J_{5a,6}$=1.3 Hz, H-5a), 2.73 (dd, 1 H, $J_{5a,5b}$=14.8, $J_{5b,6}$=6.9 Hz, H-5b), 3.86 (s, 3 H, OMe), 5.44 (m, 1 H, $J_{5a,6}$=1.6, $J_{6,7}$=2.5, $J_{5b,6}$=6.9 Hz, H-6), 5.56 (d, 1 H, $J_{6,7}$=2.6 Hz, H-7), 6.30 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.44 (d, 1 H, $J_{2',3'}$=15.9 Hz, H-2′), 6.94 and 7.54 (2d, 4 H, *J*=8.7 Hz, *p*-MeO**Ph**), 7.31 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3) 7.33–7.49 (m, 5 H, Ph), 7.77 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-3′). NOE contact: H-3 and *ortho*-H-atoms from Ph. ^{13}C NMR (62.5 MHz, CDCl$_3$): δ 40.2 (C-5), 55.4 (OMe), 78.8 (C-6), 88.1 (C-7), 113.8 (C-4), 114.6 (C-2′), 125.0 (C-2), 114.4, 125.4, 125.5, 126.8, 128.4, 128.7, 130.0, 137.9, 161.6 (2×Ph), 145.9 (C-3′), 151.1 (C-3), 166.6 (C-1′), 169.6 (C-1). HRMS (ESI): *m/z* 393.1328 (M[+]+H), calcd for C$_{23}$H$_{21}$O$_6$: 393.1333; *m/z* 410.1589 (M[+]+NH$_4$), calcd for C$_{23}$H$_{24}$NO$_6$: 410.1598; *m/z* 415.1147 (M[+]+Na), calcd for C$_{23}$H$_{20}$NaO$_6$: 415.1152.

### 4.1.6.2. (4R)-6-O-(4-methoxycinnamoyl)-(+)-crassalactone D (2c)

Yield 60%. Colourless syrup, $[\alpha]_D$=+29.0 (*c* 0.1, CHCl$_3$), R$_f$=0.38 (7:3 light petroleum/EtOAc). IR (film): $\nu_{max}$ 1775 and 1712 (C=O). ^{1}H NMR (250 MHz, CDCl$_3$): δ 2.47 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5a,6}$=4.2 Hz, H-5a), 2.89 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5b,6}$=5.7 Hz, H-5b), 3.87 (s, 3 H, OMe), 5.41 (d, 1 H, $J_{6,7}$=3.1 Hz, H-7), 5.52 (m, 1 H, H-6), 6.21 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.37 (d, 1 H, $J_{2',3'}$=15.9 Hz, H-2′), 6.94 and 7.52 (2×d, 4 H, *J*=8.7 Hz, *p*-MeO**Ph**), 7.30–7.65 (m, 6 H, H-3 and Ph), 7.72 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-3′). ^{13}C NMR (62.5 MHz, CDCl$_3$): δ 39.0 (C-5), 55.4 (OMe), 79.7 (C-6), 87.7 (C-7), 114.2 (C-2′), 114.3 (C-4), 122.8 (C-2), 114.4, 125.7, 125.8, 126.6, 128.2, 130.0, 138.0, 161.8 (2×Ph), 146.0 (C-3′), 152.8 (C-3), 166.4 (C-1′), 169.6 (C-1). HRMS (ESI): *m/z* 393.1319 (M[+]+H), calcd for C$_{23}$H$_{21}$O$_6$: 393.1333; *m/z* 410.1581 (M[+]+NH$_4$), calcd for C$_{23}$H$_{24}$NO$_6$: 410.1598; *m/z* 415.1139 (M[+]+Na), calcd for C$_{23}$H$_{20}$NaO$_6$: 415.1152.

### 4.1.6.3. (7S)-6-O-(4-methoxycinnamoyl)-(−)-crassalactone D (3c)

Yield 86%. Colourless syrup, $[\alpha]_D$=−14.0 (*c* 0.1, CHCl$_3$), R$_f$=0.49 (3:2 EtOAc/light petroleum). IR (film): $\nu_{max}$ 1771 and 1717 (C=O). ^{1}H NMR (250 MHz, CDCl$_3$): δ 2.64 (d, 1 H, $J_{5a,5b}$=14.6, H-5a), 2.79 (dd, 1 H, $J_{5a,5b}$=14.6, $J_{5b,6}$=5.3 Hz, H-5b), 3.83 (s, 3 H, OMe), 5.52 (d, 1 H, $J_{6,7}$=4.5 Hz, H-7),

5.81 (t, 1 H, $J$=4.8 Hz, H-6), 6.06 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2′), 6.26 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.88 (d, 2 H, $J$=8.8 Hz, $p$-MeO**Ph**). 7.23 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.25–7.52 (m, 8 H, Ph and H-3′). ^{13}C NMR (62.5 MHz, CDCl$_3$): δ 42.3 (C-5), 55.3 (OMe), 72.8 (C-6), 87.6 (C-7), 113.6 (C-4), 114.5 (C-2′), 124.5 (C-2), 114.2, 126.9, 127.4, 127.7, 128.3, 128.4, 129.9, 130.4, 134.9, 161.5 (2×Ph), 145.3 (C-3′), 151.9 (C-3), 166.0 (C-1′), 170.0 (C-1). HRMS (ESI): $m/z$ 393.1327 (M$^+$+H), calcd for C$_{23}$H$_{21}$O$_6$: 393.1333; $m/z$ 410.1588 (M$^+$+NH$_4$), calcd for C$_{23}$H$_{24}$NO$_6$: 410.1598; $m/z$ 415.1139 (M$^+$+Na), calcd for C$_{23}$H$_{20}$NaO$_6$: 415.1152.

### 4.1.6.4. (4R,7S)-6-O-(4-methoxycinnamoyl)-(+)-crassalactone D (4c)

Yield 77%. Colourless syrup, [α]$_D$=+17.5 ($c$ 0.2, CHCl$_3$), R$_f$=0.68 (3:2 light petroleum/EtOAc). IR (film): ν$_{max}$ 1775 and 1710 (C=O). ^{1}H NMR (250 MHz, CDCl$_3$): δ 2.61 (dd, 1 H, $J_{5a,5b}$=15.2, $J_{5a,6}$=2.6 Hz, H-5a), 3.01 (dd, 1 H, $J_{5a,5b}$=15.2, $J_{5b,6}$=6.3 Hz, H-5b), 3.84 (s, 3 H, OMe), 5.58 (d, 1 H, $J_{6,7}$=4.4 Hz, H-7), 5.86 (ddd, 1 H, $J_{5a,6}$=2.6, $J_{6,7}$=4.4, $J_{5b,6}$=6.5 Hz, H-6), 6.01 (d, 1 H, $J_{2',3'}$=15.9 Hz, H-2′), 6.24 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.88 (d, 2 H, $J$=8.8 Hz, $p$-MeO**Ph**), 7.27–8.42 (m, 9 H, 2×Ph, H-3 and H-3′). ^{13}C NMR (62.5 MHz, CDCl$_3$): δ 42.1 (C-5), 55.3 (OMe), 74.3 (C-6), 85.0 (C-7), 112.7 (C-4), 114.2 (C-2′), 123.9 (C-2), 114.3, 126.7, 126.9, 128.1, 128.3, 129.8, 134.3, 161.6 (2×Ph), 145.3 (C-3′), 151.7 (C-3), 165.8 (C-1′), 169.6 (C-1). HRMS (ESI): $m/z$ 393.1324 (M$^+$+H), calcd for C$_{23}$H$_{21}$O$_6$: 393.1333; $m/z$ 410.1594 (M$^+$+NH$_4$), calcd for C$_{23}$H$_{24}$NO$_6$: 410.1598; $m/z$ 415.1142 (M$^+$+Na), calcd for C$_{23}$H$_{20}$NaO$_6$: 415.1152.

### 4.1.7. General procedure for the preparation of 4-fluorocinnamoates 1d–4d

To a solution **1–4** (1 equiv)[†] in dry CH$_2$Cl$_2$ (0.04–0.05 M) were added 4-fluorocinnamic acid (2.3 equiv), DMAP (4.6 equiv) and DCC (2.4 equiv). The mixture was stirred at room temperature for 4–5 h,[‡] then poured into water and extracted with CH$_2$Cl$_2$. The combined extracts were washed with 10% aq NaCl, dried and evaporated. The residue was purified by flash column chromatography (3:2 light petroleum/EtOAc for **3d**, 4:1 light petroleum/EtOAc for **4d**) or by flash chromatography (7:3 light petroleum/EtOAc) followed by preparative TLC (7:3 light petroleum/EtOAc for **1d** and **2d**).

### 4.1.7.1. 6-O-(4-fluorocinnamoyl)-(+)-crassalactone D (1d)

Yield 36%. Colourless needles, mp 152–155 °C (CH$_2$Cl$_2$/hexane), [α]$_D$=+62.0 ($c$ 0.1, CHCl$_3$), R$_f$=0.34 (7:3 light petroleum/EtOAc). IR (film): ν$_{max}$ 1775 and 1715 (C=O). ^{1}H NMR (250 MHz,

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[‡] For **3** additional portions of reagents have been added: 4-fluorocinnamic acid (1.2 equiv), DMAP (2.3 equiv) and DCC (2.3 equiv) and stirring continued for additional 1 h at room temperature.

CDCl$_3$): δ 2.46 (bd, 1 H, $J_{5a,5b}$=14.8 Hz, H-5a), 2.73 (dd, 1 H, $J_{5a,5b}$=14.8, $J_{5b,6}$=6.8 Hz, H-5b), 5.45 (m, 1 H, H-6), 5.57 (d, 1 H, $J_{6,7}$=2.4 Hz, H-7), 6.31 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.51 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2′), 7.31 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.03–7.64 (m, 9 H, 2×Ph), 7.78 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-3′). $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 40.2 (C-5), 79.0 (C-6), 88.1 (C-7), 113.7 (C-4), 116.97 and 117.0 (C-2′, $J_{2',F}$=1.8 Hz), 125.0 (C-2), 116.0 and 116.3 (C-3″, $J_{3'',F}$=18.8 Hz), 125.4, 128.4, 128.8, 130.2, 130.3, 137.8 (2×Ph), 162.1 and 166.5 (C-4″, $J_{4'',F}$=275.0 Hz), 145.0 (C-3′), 151.1 (C-3), 166.1 (C-1′), 169.6 (C-1). HRMS (ESI): *m/z* 381.1122 (M$^+$+H), calcd for C$_{22}$H$_{18}$FO$_5$: 381.1133; *m/z* 398.1388 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$NFO$_5$: 398.1398; *m/z* 403.0943 (M$^+$+Na), calcd for C$_{22}$H$_{17}$NaFO$_5$: 403.0952.

### 4.1.7.2. (4R)-6-O-(4-fluorocinnamoyl)-(+)-crassalactone D (2d)

Yield 50%. Colourless syrup, [α]$_D$=+24.0 (*c* 0.1, CHCl$_3$), R$_f$=0.54 (7:3 light petroleum/EtOAc). IR (film): ν$_{max}$ 1776 and 1717 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.48 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5a,6}$=4.3 Hz, H-5a), 2.90 (dd, 1 H, $J_{5a,5b}$=14.3, $J_{5b,6}$=5.7 Hz, H-5b), 5.42 (d, 1 H, $J_{6,7}$=3.2 Hz, H-7), 5.54 (m, 1 H, H-6), 6.21 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 6.43 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2′), 7.35 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.08–7.64 (m, 9 H, 2×Ph), 7.73 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-3′). $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 39.1 (C-5), 79.8 (C-6), 87.6 (C-7), 114.2 (C-4), 116.5 and 116.53 (C-2′, $J_{2',F}$=1.8 Hz), 123.0 (C-2), 116.0 and 116.3 (C-3″, $J_{3'',F}$=18.8 Hz), 125.8, 128.3, 128.7, 130.1, 130.2, 137.9 (2×Ph), 162.1 and 166.2 (C-4″, $J_{4'',F}$=256.3 Hz), 145.0 (C-3′), 152.7 (C-3), 165.9 (C-1′), 169.6 (C-1). HRMS (ESI): *m/z* 381.1122 (M$^+$+H), calcd for C$_{22}$H$_{18}$FO$_5$: 381.1133; *m/z* 398.1388 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$NFO$_5$: 398.1398; *m/z* 403.0945 (M$^+$+Na), calcd for C$_{22}$H$_{17}$NaFO$_5$: 403.0952.

### 4.1.7.3. (7S)-6-O-(4-fluorocinnamoyl)-(+)-crassalactone D (3d)

Yield 73%. Colourless syrup, [α]$_D$=+60.0 (*c* 0.1, CHCl$_3$), R$_f$=0.58 (1:1 EtOAc/light petroleum). IR (film): ν$_{max}$ 1774 and 1713 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.64 (d, 1 H, $J_{5a,5b}$=14.6 Hz, H-5a), 2.79 (dd, 1 H, $J_{5a,5b}$=14.6, $J_{5b,6}$=5.3 Hz, H-5b), 5.53 (d, 1 H, $J_{6,7}$=4.4, $J_{5b,6}$=5.2 Hz, H-7), 5.81 (dd, 1 H, $J_{6,7}$=4.6 Hz, H-6), 6.11 (d, 1 H, $J_{2',3'}$=16.0 Hz, H-2′), 6.27 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 7.06 (t, 2 H, *p*-F-cinnamate), 7.24 (d, 1 H, $J_{2,3}$=5.5 Hz, H-3), 7.26–7.51 (m, 8 H, Ph and H-3′). $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 42.3 (C-5), 73.0 (C-6), 87.4 (C-7), 113.5 (C-4), 116.8 and 116.83 (C-2′, $J_{2',F}$=1.8 Hz), 124.6 (C-2), 115.8 and 116.1 (C-3″, $J_{3'',F}$=18.8 Hz), 127.4, 128.0, 128.3, 130.0, 130.36, 130.4, 134.8 (2×Ph), 161.9 and 165.9 (C-4″, $J_{4'',F}$=250.0 Hz), 144.3 (C-3′), 151.8 (C-3), 165.5 (C-1′), 170.0 (C-1). HRMS (ESI): *m/z* 398.1411 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$NFO$_5$: 398.1398; *m/z* 381.1138 (M$^+$+H), calcd for C$_{122}$H$_{18}$FO$_5$: 381.1133.

*4.1.7.4. (4R,7S)-6-O-(4-fluorocinnamoyl)-(+)-crassalactone D (4d)*

Yield 88%. Colourless syrup, $[\alpha]_D$=+34.5 (*c* 0.2, CHCl$_3$), R$_f$=0.56 (7:3 light petroleum/EtOAc). IR (film): $\nu_{max}$ 1778 and 1716 (C=O). $^1$H NMR (250 MHz, CDCl$_3$): δ 2.61 (dd, 1 H, $J_{5a,5b}$=15.2, $J_{5a,6}$=2.7 Hz, H-5a), 3.01 (dd, 1 H, $J_{5a,5b}$=15.2, $J_{5b,6}$=6.4 Hz, H-5b), 5.58 (d, 1 H, $J_{6,7}$=4.4 Hz, H-7), 5.87 (ddd, 1 H, $J_{5a,5b}$=2.7, $J_{6,7}$=4.4, $J_{5b,6}$=6.5 Hz, H-6), 6.06 (d, 1 H, $J_{2',3}$=15.9 Hz, H-2′), 6.26 (d, 1 H, $J_{2,3}$=5.5 Hz, H-2), 7.00–7.47 (m, 11 H, 2×Ph, H-3 and H-3′). $^{13}$C NMR (62.5 MHz, CDCl$_3$): δ 42.1 (C-5), 74.5 (C-6), 84.9 (C-7), 112.6 (C-4), 116.46 and 116.49 (C-2′, $J_{2',F}$=1.9 Hz), 124.0 (C-2), 115.9 and 116.2 (C-3″, $J_{3'',F}$=18.8 Hz), 126.9, 128.1, 128.3, 129.9, 130.0, 134.2 (2×Ph), 162.0 and 165.99 (C-4″, $J_{4'',F}$=249.4 Hz), 144.2 (C-3′), 151.5 (C-3), 166.0 (C-1′), 169.5 (C-1). HRMS (ESI): *m/z* 381.1128 (M$^+$+H), calcd for C$_{22}$H$_{18}$FO$_5$: 381.1133; *m/z* 398.1392 (M$^+$+NH$_4$), calcd for C$_{22}$H$_{21}$FNO$_5$: 398.1398; *m/z* 403.0947 (M$^+$+Na), calcd for C$_{22}$H$_{17}$FNaO$_5$: 403.0952.

*4.2. X-ray Crystal Structure Analysis*

Diffraction experiments were performed on an Oxford Diffraction Gemini S diffractometer. Crystal structures were solved by SHELXT [26] or SIR92 [27] and refined with SHELXL [28]. See the Supplementary data for details. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre. CCDC Numbers: 1530428 (**4**), 1530429 (**3**), 1530430 (**2a**), 1530431 (**1b**), 1530432 (**1c**), 1530433 (**1d**) and 1530434 (**4a**). These data are available free of charge via www.ccdc.cam.ac.uk/data_request/cif.

*4.3. MTT assay*

The colorimetric MTT assay was carried out following reported procedure [18].

*4.4. Cell cycle analysis*

K562 cells were treated with tested compounds for 72 h at their IC$_{50}$ concentrations. After treatment, K562 cells were washed in cold PBS and incubated for 30 min in 70% ethanol on ice, centrifuged and incubated with 500 µL Rnase A (100 units/mL) and 500 µL propidium iodide (400 µL/mL) for 30 min at 37 °C. Cell cycle was analyzed by FACS Calibur E440 (Becton Dickinson) flow cytometer and the Cell Quest software. Results were presented as percentage of cell cycle phases.

*4.5. Detection of apoptosis*

Apoptosis of K562 cells was evaluated with an Annexin V-FITC detection kit. Treated cells from each sample were collected (800 rpm/5 min, Megafuge 1.0R, Heraeus, Thermo Fisher Scientific) and pellet was re-suspended in 1mL of phosphate buffer (PBS, pH 7.2). K562 cells were washed twice with cold PBS and then re-suspended in binding buffer to reach the concentration of $1 \times 10^6$ cells/mL. The cell suspension (100 µL) was transferred to 5mL culture tubes and mixed with Annexin V (5 µL) and propidium iodide (5 µL). The cells were gently vortexed and incubated for 15 min at 25 °C. After incubation, 400 mL of binding buffer was added to each tube and suspension was analyzed after 1 h on FACS Calibur E440 (Becton Dickinson) flow cytometer. Results were presented as percent of Annexin V positive gated cells. Percentage of specific apoptosis was calculated according to Bender et al. [22].

*4.6. Western blot*

For the Western blot, 50 µg of proteins per sample were separated by electrophoresis and electro-transferred to a PVDF membrane Hybond-P and then blotted with primary antibodies against Bcl-2, Bax, caspase 3 and PARP. β-Actin was used as internal control. Proteins were detected by an enhanced chemiluminescence (ECL Plus) kit that includes peroxidase-labelled donkey anti-rabbit and sheep anti-mouse secondary antibodies. Blots were developed with an ECL Plus detection system and recorded on the Amersham Hyperfilm. Images of protein expression were analyzed in ImageJ computer program (NIH Image, http://imagej.nih.gov) after minor levels adjustments. Expression of proteins was measured by densitometry and compared with control sample.

**Acknowledgement**

**Supplementary data**

Contains the results of: X-ray crystal structure determination, SAR analysis, flow cytometry and Western blot analysis. Copies of $^1$H and $^{13}$C NMR spectra of final products are also disclosed. This material is available free of charge via the Internet.

**References**

[1] P. Tuchinda, B. Munyoo, M. Pohmakotr, P. Thinapong, S. Sophasan, T. Santisuk, V. Reutrakul, Cytotoxic styryl-lactones from the leaves and twigs of *Polyalthia crassa*, J. Nat. Prod. 69 (2006) 1728–1733.

[2] E. Pavlakos, T. Georgiou, M. Tofi, T. Montagnon, G. Vassilikogiannakis, γ-Spiroketal γ-lactones from 2-(γ-hydrokylalkyl)furans: syntheses of *epi*-pyrenolides D and crassalactone D, Org. Lett. 11 (2009) 4556–4559.

[3] Z. Yang, P. Tang, J.F. Gauuan, B.F. Molino, Asymmetric total synthesis of (+)-crassalactone D, J. Org. Chem. 74 (2009) 9546–9549.

[4] R. Galán-Fernádez, D. Clemente-Tejeda, F.A. Bermejo, Oxidative cyclization of γ-alkylidene butenolides. Stereoselective preparation of spirolactones, Arkivoc, ix (2012), 171–184.

[5] A part of this work appeared as a preliminary communication: V. Popsavin, I. Kovačević, G. Benedeković, M. Popsavin, V. Kojić, G. Bogdanović, Divergent synthesis of cytotoxic styryl lactones related to goniobutenolides A and B, and to crassalactone D, Org. Lett. 14 (2012) 5956–5959.

[6] P. De, M. Baltas, F. Bedos-Belval, Cinnamic acid derivatives as anticancer agents – a review, Curr. Med. Chem. 18 (2011) 1672–1703.

[7] C. Viegas-Junior, A. Danuello, V. da Silva Bolzani, E.J. Barreiro; C.A.M. Fraga, Molecular hybridization: A useful tool in the design of new drug prototypes, Curr. Med. Chem. 14 (2007) 1829–1852.

[8] G. Benedeković, J. Francuz, I. Kovačević, M. Popsavin, B. Srećo Zelenović, V. Kojić, G. Bogdanović, V. Divjaković, V. Popsavin, Conformationally constrained goniofufurone mimics as inhibitors of tumour cells growth: design, synthesis and SAR study, Eur. J. Med. Chem. 82 (2014) 449–458.

[9] G. Benedeković, M. Popsavin, J. Francuz, I. Kovačević, V. Kojić, G. Bogdanović, V. Divjaković, V. Popsavin, Design, synthesis and SAR analysis of antitumour styryl lactones related to (+)-crassalactones B and C, Eur. J. Med. Chem. 87 (2014) 237–247.

[10] I. Kovačević, M. Popsavin, G. Benedeković, V. Kojić, D. Jakimov, M.V. Rodić, T. Srdić-Rajić, G. Bogdanović, V. Divjaković, V. Popsavin, Synthesis and antiproliferative activity of goniobutenolides A and B, 5-halogenated crassalactone D derivatives and the corresponding 7-epimers, Eur. J. Med. Chem. 108 (2016) 594–604.

[11] J.E. Harvey, S.A. Raw, R.J.K. Taylor, A versatile and stereocontrolled route to pyranose and furanose *C*-glycosides, Org. Lett. 6 (2004) 2611–2614.

[12] S. Valverde, M. Martin-Lomas, B. Herradon, S. Garcia-Ochoa, The reaction of carbohydrate-derived alkoxyaldehydes with methoxycarbonylmethylenetriphenylphosphorane: stereoselective synthesis of β-unsaturated esters, Tetrahedron 43 (1987) 1895–1901.

[13] K.R.C. Prakash, S. Prahlada Rao, Ethoxycarbonylmethylenetriphenylphosphorane in carbohydrate chemistry: an easy access to functionalized 2-deoxy-3,6-anhydrohexono-1,4-lactones, Tetrahedron Lett. 32 (1991) 7473–7476.

[14] K.R.C. Prakash, S. Prahlada Rao, Ethoxycarbonylmethylenetriphenylphosphorane in carbohydrate chemistry, part III: a short and highly efficient synthesis of (−)-epigoniofufuron, Synlett 2 (1993) 123–124.

[15] J.E. Aho, P.M. Pihko, T.K. Rissa, Nonanomeric spiroketals in natural products: structures, sources, and synthetic strategies, Chem. Rev. 105 (2005) 4406–4440.

[16] A.J. Kirby, The anomeric effect and related stereoelectronic effects at oxygen, in: Hafner, K., Rees, C.W., Trost, B.M., Lehn, J.-M., von Rague Schleyer, P., Zahradnik, R. (Eds), Reactivity and structure concepts in organic chemistry, Vol. 15, Springer-Verlag, Berlin, Heidelberg, New York, 1983, pp. 1–147.

[17] B. Neises, W. Steglich, Esterification of carboxylic acids with dicylohexylcarbodiimide/4-dimethylaminopyridine: tert-butyl ethyl fumarate, Org. Synth. 63 (1985) 183–186.

[18] D.A. Scudiero, R.H. Shoemaker, K.D. Paull, A. Monks, S. Tierney, T.H. Nofziger, M.J. Currens, D. Seniff, M.R. Boyd, Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines, Cancer. Res. 48 (1988) 4827–4833.

[19] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.

[20] G. Housman, S. Byler, S. Heerboth, K. Lapinska, M. Longrace, N. Snyder, S. Sarkar, Drug resistance in cancer: an overview, Cancers 6 (2014) 1769–1792.

[21] C.-Y. Choo, N. Abdullah, M. Diederich, Cytotoxic activity and mechanism of action of metabolites from the *Goniothalamus* genus, Phytochem. Rev. 13 (2014) 835–851.

[22] A. Bender, D. Opel, I. Naumann, R. Kappler, L. Friedman, D. Von Schwinitz, K.-M. Debatin, S. Fulda, PI3K inhibitors prime neuroblastoma cells for chemotherapy by shifting the balance towards pro-apoptotic Bcl-2 proteins and enhanced mitochondrial apoptosis, Oncogene, 30 (2011) 494–503.

[23] P.E. Czabotar, G. Lessene, A. Strasser, J.M. Adams, Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy, Nat. Rev. Mol. Cell Biol. 15 (2014) 49–63.

[24] G.S. Salvesen, Caspases: opening the boxes and interpreting the arrows, Cell Death Differ. 9 (2002) 3–5.

[25] C. Soldani, M.C. Lazzè, M.G. Bottone, G. Tognon, M. Biggiogera, C.E. Pellicciari, A.I. Scovassi, Poly(ADP-ribose) polymerase cleavage during apoptosis: when and where? Exp. Cell. Res. 269 (2001) 193–201.

[26] G.M. Sheldrick SHELXT – Integrated space-group and crystal structure determination, Acta Crystallogr. Sect. A 71 (2015) 3–8.

[27] A. Altomare, G. Cascarano, C. Giacovazzo, A. Gualardi, Completion and refinement of crystal structures with SIR92, J. Appl. Crystallogr. 26 (1993) 343–350.

[28] G.M. Sheldrick, Crystal structure refinement with SHELXL, Acta Crystallogr. Sect. C 71 (2015) 3–8.

**CAPTIONS**

**Figure 1**. Chemical structures of crassalactone D (**1**), its epimers **2**–**4** and 6-*O*-cinnamoyl derivatives **1a**–**d**, **2a**–**d**, **3a**–**d** and **4a**–**d**.

**Figure 2.** Results of Western blot analysis after treatment of K562 cells with the synthesized compounds.

**Scheme 1**. Reagents and conditions: (a) 90% aq TFA, 0 °C for 0.5 h, then rt, 1 h for **5**, 1.5 h for **7**; (b) Ph$_3$P=CHCO$_2$Me, dry MeOH, 0 °C for 0.5, then rt, 21 h for **6**, 47 h for **8**, 51% (2:1 mixture of **2** and **1**), 29% of **3**, 38% of **4**; (c) Ph$_3$P=CHCO$_2$Et, dry MeOH, 0 °C for 1.5 h, then rt, 25 h for **8**, 10% of **3**, 37% of **4**; (d) TFA, CHCl$_3$, rt, 63 h for 1:2 mixture of **1** and **2**, 99% of 2:1 mixture of **1** and **2**, 23 h for **4**, 1:1 mixture of **3** and **4**.
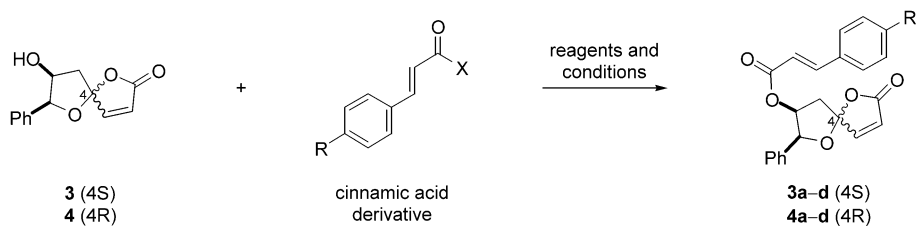
**TABLES**

**Table 1.** Preparation of cinnamic ester derivatives **1a–d** and **2a–d**.
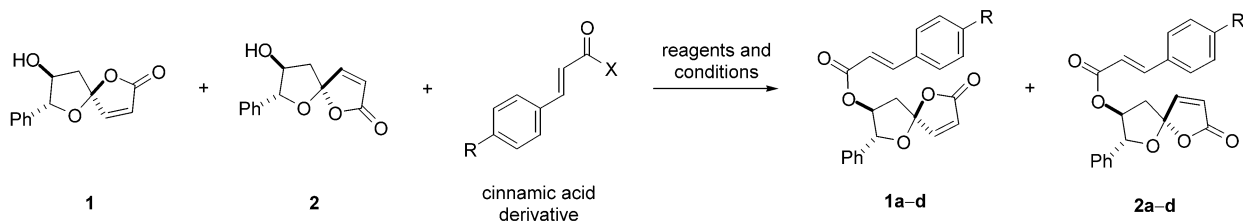


| Entry | Starting compound | Cinnamic acid derivative | Reagents and conditions | Products (isolated yield, %) |
|---|---|---|---|---|
| 1 | **1** + **2** (1:2 mixture) | R = H, X = Cl | DMAP, CH$_2$Cl$_2$, 3 h, 0 °C, 20 h, rt | **1a** (34), **2a** (38), R = H |
| 2 | **1** + **2** (1:2 mixture) | R = NO$_2$, X = Cl | DMAP, CH$_2$Cl$_2$, 3 h, 0 °C | **1b** (29), **2b** (56), R = NO$_2$ |
| 3 | **1** + **2** (1:2 mixture) | R = OMe, X = OH | DMAP, DCC, CH$_2$Cl$_2$, 5 h, rt | **1c** (32), **2c** (60), R = OMe |
| 4 | **1** + **2** (1:2 mixture) | R = F, X = OH | DMAP, DCC, CH$_2$Cl$_2$, 5 h, rt | **1d** (36), **2d** (50), R = F |

**Table 2.** Preparation of cinnamic ester derivatives **3a–d** and **4a–d**.



**3** (4S)
**4** (4R)

cinnamic acid derivative

**3a–d** (4S)
**4a–d** (4R)

| Entry | Starting compound | Cinnamic acid derivative | Reagents and conditions | Products (isolated yield, %) |
|---|---|---|---|---|
| 1 | | R = H, X = Cl | DMAP, CH$_2$Cl$_2$, 3 h, 0 °C, 45 min, rt | **3a** (68), R = H |
| 2 | | R = NO$_2$, X = Cl | DMAP, CH$_2$Cl$_2$, 3.5 h, 0 °C | **3b** (74), R = NO$_2$ |
| 3 | **3** | R = OMe, X = OH | DMAP, DCC, CH$_2$Cl$_2$, 3.5 h, rt | **3c** (86), R = OMe |
| 4 | | R = F, X = OH | DMAP, DCC, CH$_2$Cl$_2$, 5.5 h, rt | **3d** (73), R = F |
| 5 | | R = H, X = Cl | DMAP, CH$_2$Cl$_2$, 3 h, 0 °C, 22.5 h, rt | **4a** (62), R = H |
| 6 | | R = NO$_2$, X = Cl | DMAP, CH$_2$Cl$_2$, 3.5 h, 0 °C | **4b** (89), R = NO$_2$ |
| 7 | **4** | R = OMe, X = OH | DMAP, DCC, CH$_2$Cl$_2$, 3 h, rt | **4c** (77), R = OMe |
| 8 | | R = F, X = OH | DMAP, DCC, CH$_2$Cl$_2$, 4 h, rt | **4d** (88), R = F |

**Table 3.** *In vitro* cytotoxicity of natural product **1**, its analogues **2–4**, 6-*O*-cinnamoyl derivatives **1a–d**, **2a–d**, **3a–d**, **4a–d** and DOX.

| Compd | IC$_{50}$ (μM)[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | K562 | HL-60 | Jurkat | Raji | MCF-7 | MDA-MB 231 | HeLa | A549 | MRC-5 |
| **1** | 0.12±0.01 | 1.06±0.69 | 0.21±0.04 | 2.34±0.58 | 11.64±0.53 | 1.89±0.22 | 0.34±0.01 | 28.99±1.25 | >100 |
| **1a** | 20.67±1.21 | 10.02±2.79 | 0.21±0.01 | 6.68±1.25 | 1.65±0.21 | 5.45±1.21 | 1.15±0.15 | 14.58±1.52 | >100 |
| **1b** | 5.64±0.11 | 2.24±0.69 | 31.25±2.54 | >100 | 8.58±0.31 | >100 | 2.22±0.08 | 45.36±3.41 | >100 |
| **1c** | 2.12±0.25 | 8.67±0.79 | 58.14±3.35 | 89.68±4.56 | 3.14±0.11 | >100 | 3.34±0.03 | 33.56±1.58 | >100 |
| **1d** | 8.45±0.99 | 24.13±3.54 | 15.64±1.54 | 34.12±3.41 | 9.65±0.05 | >100 | 6.12±0.65 | >100 | >100 |
| **2** | 0.54±0.11 | 1.02±0.87 | 0.34±0.02 | 5.97±1.12 | 3.57±0.18 | >100 | 2.12±0.32 | 21.33±2.54 | >100 |
| **2a** | 20.53±2.25 | 5.61±0.67 | 87.63±2.99 | 2.38±0.56 | 0.58±0.02 | 3.45±0.89 | 0.58±0.01 | 28.07±2.84 | >100 |
| **2b** | 7.45±1.25 | 1.32±0.54 | 86.54±3.54 | >100 | 12.46±1.21 | >100 | 9.45±1.52 | 56.21±3.41 | >100 |
| **2c** | 1.11±0.98 | 6.37±0.69 | 21.14±2.07 | 99.78±5.84 | 2.22±0.21 | >100 | 3.64±0.38 | 23.14±1.42 | >100 |
| **2d** | 1.05±0.79 | 4.48±0.77 | 22.17±3.52 | 55.36±3.12 | 21.48±2.54 | >100 | 2.21±0.12 | 25.47±0.52 | >100 |
| **3** | 1.21±0.25 | 6.11±0.54 | 1.01±0.11 | 8.84±0.88 | 11.02±0.45 | 45.13±4.32 | 35.41±2.55 | 35.21±2.45 | >100 |
| **3a** | 4.62±0.69 | 3.41±0.02 | 5.63±0.66 | 9.68±0.87 | 5.34±0.88 | 5.23±0.89 | 14.46±0.84 | 1.02±0.11 | >100 |
| **3b** | 14.32±2.58 | 4.32±0.32 | 10.22±0.98 | 11.84±2.02 | 7.55±0.65 | 4.48±0.15 | 11.48±0.54 | 3.12±0.12 | >100 |
| **3c** | 12.78±1.64 | 8.67±0.78 | 14.22±1.32 | 8.69±1.21 | 11.25±1.08 | 2.64±0.11 | 12.78±1.32 | 1.58±0..05 | >100 |
| **3d** | 9.05±2.54 | 12.02±0.65 | 15.36±1.58 | 17.33±1.32 | 15.63±2.51 | 1.02±0.02 | 11.98±0.12 | 1.21±0.09 | >100 |
| **4** | 0.54±0.04 | 0.19±0.05 | 0.96±0.05 | 2.69±0.56 | 1.97±0.89 | >100 | 4.61±0.41 | 31.37±2.54 | >100 |
| **4a** | 4.65±0.94 | 9.65±0.55 | 1.02±0.07 | 2.28±0.24 | 11.44±1.23 | 3.64±0.38 | 58.45±4.22 | 0.25±0.01 | >100 |
| **4b** | 2.22±0.54 | 4.36±0.05 | 4.03±0.36 | 1.64±0.12 | 4.31±0.16 | 1.25±0.15 | 24.77±3.52 | 0.85±0.03 | >100 |
| **4c** | 1.02±0.56 | 2.25±0.09 | 2.54±0.77 | 3.05±0.01 | 3.25±0.14 | 2.69±0.22 | 9.94±1.52 | 0.45±0.01 | >100 |
| **4d** | 5.36±0.87 | 1.02±0.12 | 4.63±0.87 | 5.37±0.21 | 2.31±0.12 | 7.54±0.65 | 8.45±0.87 | 1.17±0.21 | >100 |
| DOX | 0.25±0.02 | 0.92±0.09 | 0.03±0.005 | 2.98±0.98 | 0.20±0.02 | 0.09±0.002 | 0.07±0.01 | 4.91±0.31 | 0.10±0.01 |

[a] IC$_{50}$ is the concentration of compound required to inhibit cell growth by 50% compared to an untreated control. The values are means of three independent experiments done in quadruplicates. Coefficients of variation were <10%

**Table 4**. (A) Influence of synthesized compound on the K562 cell cycle; (B) percentage of specific apoptosis and necrosis induced with the synthesized compounds in the K562 cell culture.

| Compound | (A) Distribution of K562 cells in cell cycle phases (%) | | | | (B) Type of cell death | |
|---|---|---|---|---|---|---|
| | Sub G1 | G0/G1 | S | G2/M | Specific apoptosis (%) | Specific necrosis (%) |
| control | 1.48 | 38.48 | 41.03 | 19.01 | – | – |
| **1** | 1.60 | 32.52 | 59.02 | 6.86 | 22.60 | 1.08 |
| **1a** | 28.63 | 16.04 | 31.55 | 23.78 | 83.35 | 4.06 |
| **1b** | 2.13 | 48.17 | 28.04 | 21.66 | 0.75 | 3.40 |
| **1c** | 3.39 | 21.08 | 41.08 | 34.45 | 37.78 | 3.86 |
| **1d** | 2.02 | 40.27 | 28.31 | 29.40 | 8.05 | 3.28 |
| **2** | 1.26 | 39.26 | 35.20 | 24.28 | 11.74 | 4.80 |
| **2a** | 56.93 | 23.10 | 14.63 | 5.34 | 86.01 | 4.12 |
| **2b** | 2.93 | 33.85 | 38.08 | 25.14 | 10.54 | 2.18 |
| **2c** | 1.57 | 30.72 | 42.64 | 25.07 | 15.35 | 3.15 |
| **2d** | 2.61 | 41.18 | 33.71 | 22.50 | 4.02 | 3.58 |
| **3** | 1.09 | 15.18 | 17.17 | 66.56 | 7.78 | 2.84 |
| **3a** | 5.69 | 21.01 | 38.09 | 35.21 | 81.79 | 1.97 |
| **3b** | 20.43 | 30.42 | 29.17 | 19.98 | 81.97 | 0.64 |
| **3c** | 3.41 | 41.60 | 27.14 | 27.85 | 2.28 | 3.76 |
| **3d** | 14.87 | 23.20 | 37.11 | 24.82 | 85.69 | 3.78 |
| **4** | 1.10 | 28.98 | 52.34 | 17.58 | 10.05 | 0.67 |
| **4a** | 12.49 | 27.11 | 42.16 | 18.24 | 6.76 | 28.29 |
| **4b** | 40.08 | 30.15 | 22.70 | 7.07 | 70.03 | 1.76 |
| **4c** | 3.23 | 47.26 | 29.40 | 20.11 | 2.36 | 1.60 |
| **4d** | 3.99 | 37.01 | 30.34 | 28.66 | 18.71 | 1.22 |

**FIGURES**
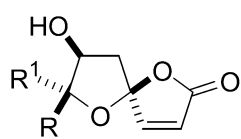


**1** R = H, R$^1$ = Ph
**3** R = Ph, R$^1$ = H
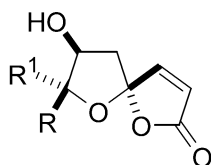
**2** R = H, R$^1$ = Ph
**4** R = Ph, R$^1$ = H
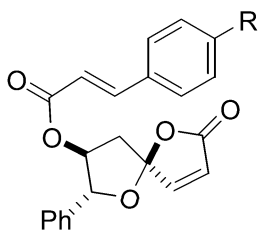
| R | H | NO$_2$ | OMe | F |
|---|---|---|---|---|
| | 1a | 1b | 1c | 1d |

| R | H | NO$_2$ | OMe | F |
|---|---|---|---|---|
| | 2a | 2b | 2c | 2d |

| R | H | NO$_2$ | OMe | F |
|---|---|---|---|---|
| | 3a | 3b | 3c | 3d |

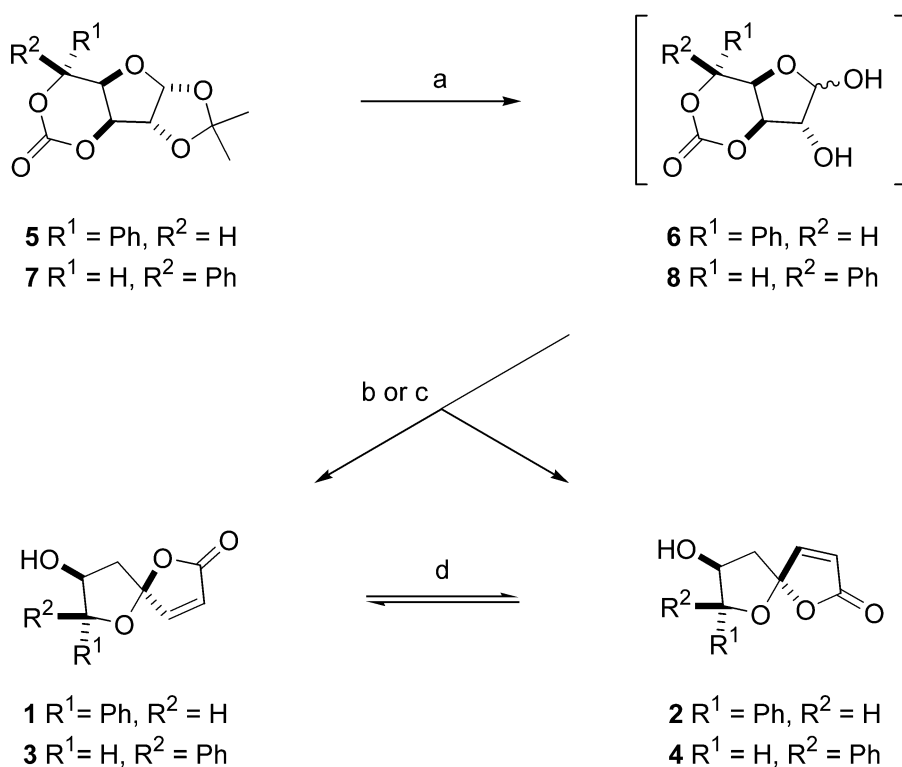| R | H | NO$_2$ | OMe | F |
|---|---|---|---|---|
| | 4a | 4b | 4c | 4d |
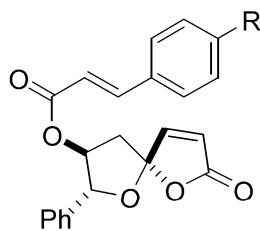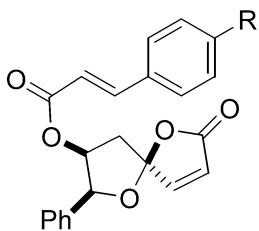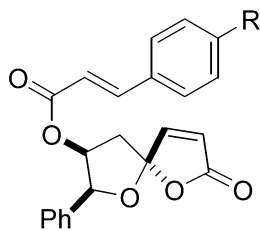
**Figure 1**. Chemical structures of crassalactone D (**1**), its epimers **2–4** and 6-*O*-cinnamoyl derivatives **1a–d**, **2a–d**, **3a–d** and **4a–d**.

**Figure 2.** Results of Western blot analysis after treatment of K562 cells with the synthesized compounds.

**SCHEMES**

**5** $R^1$ = Ph, $R^2$ = H
**7** $R^1$ = H, $R^2$ = Ph

**6** $R^1$ = Ph, $R^2$ = H
**8** $R^1$ = H, $R^2$ = Ph

**1** $R^1$ = Ph, $R^2$ = H
**3** $R^1$ = H, $R^2$ = Ph

**2** $R^1$ = Ph, $R^2$ = H
**4** $R^1$ = H, $R^2$ = Ph

**Scheme 1**. Reagents and conditions: (a) 90% aq TFA, 0 °C for 0.5 h, then rt, 1 h for **5**, 1.5 h for **7**; (b) $Ph_3P=CHCO_2Me$, dry MeOH, 0 °C for 0.5, then rt, 21 h for **6**, 47 h for **8**, 51% (2:1 mixture of **2** and **1**), 29% of **3**, 38% of **4**; (c) $Ph_3P=CHCO_2Et$, dry MeOH, 0 °C for 1.5 h, then rt, 25 h for **8**, 10% of **3**, 37% of **4**; (d) TFA, $CHCl_3$, rt, 63 h for 1:2 mixture of **1** and **2**, 99% of 2:1 mixture of **1** and **2**, 23 h for **4**, 1:1 mixture of **3** and **4**.