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Research paper

Synthesis and antiproliferative activity of goniobutenolides A and B, 5-halogenated crassalactone D derivatives and the corresponding 7-epimers

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ABSTRACT

A new synthesis of goniobutenolides A (**1**) and B (**2**) and the corresponding 7-epimers has been achieved starting from diacetone D-glucose. The key step of the synthesis is a new one-pot sequence that commenced with *Z*-selective Wittig (or Horner-Wadsworth-Emmons) olefination, followed by successive γ-lactonisation and β-elimination. The above-mentioned unsaturated lactones were then converted to the corresponding 5-halogenated crassalactone D derivatives by using the appropriate haloetherification protocol. The most of synthesized compounds exhibited potent cytotoxic activities against a panel of tumour cell lines. The main structural features responsible for their antitumour potency have been revealed by means of SAR analysis. Flow cytometry data suggested that cytotoxic effects of these compounds in the culture of K562 cells might be mediated by apoptosis, additionally revealing that these molecules induced changes in cell cycle distribution of these cells. Results of semi-quantitative Western blot analysis indicate that the most of synthesized compounds induce apoptosis in a caspase-dependent manner.

1. Introduction

Goniobutenolide A (**1**), goniobutenolide B (**2**) and crassalactone D (**3**) (Fig. 1) are products of secondary metabolism of Annonaceae plant family, the largest family in Magnoliales. Goniobutenolides A and B have been isolated from the stem bark of *Gonithalamus giganteus* by brine shrimp lethality-directed fractionation of the ethanolic extract and have been found to exhibit cytotoxicity toward few cancer cell lines [1]. Although these compounds were the targets of synthetic efforts by several groups [2–9], their biological activities were not investigated in details. Tuchinda and co-workers isolated crassalactone D (**3**) from the tropical plant *Polyalthia Crassa* and evaluated its cytotoxicity against several mammalian cancer cell lines [10]. In 2009 two research groups reported independently total synthesis of **3** [11,12]. Recent report disclosed by Bermejo et al. [13] describes a bromoetherification of 6-*epi*-goniobutenolides A and B that afforded a mixture of isomeric bromo-spirolactones. This finding prompted us to extend this reaction to the natural goniobutenolides A (**1**) and B (**2**), as well as to the corresponding 7-epimers, in order to prepare novel crassalactone D and 7-*epi*-crassalactone D 5-halogen-derivatives for biological testing. The additional reason for carrying out of such a research is based on recent findings, which showed that some halogenated natural products exhibit a variety of biological activities [14,15].

Herein, we disclose full details on the synthesis of **1** and **2** [16], as well as their conversion to novel halogenated spiro-lactones **3a**, **3b**, **4a**–**d** including a number of selected 7-epimers (**5**–**8**, **7a**, **7b**, **8a**–**c**). The effects of these compounds to the proliferation of certain human tumour cell lines, including their effects on the K562 cell cycle and their apoptosis inducing properties in the same

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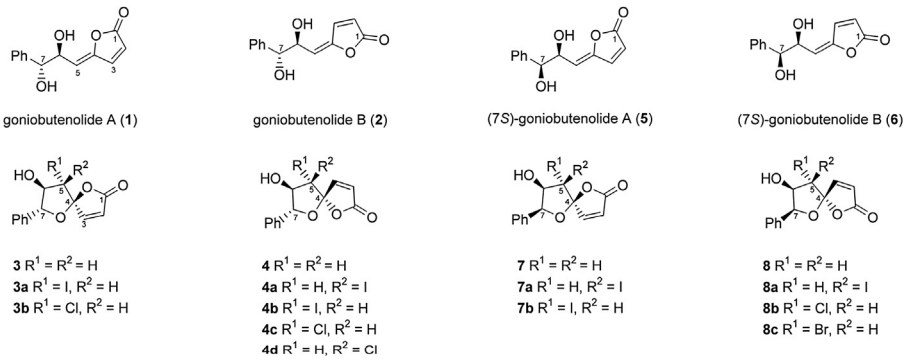


Fig. 1. Chemical structures of goniobutenolide A (**1**), goniobutenolide B (**2**), crassalactone D (**3**), (4*R*)-crassalactone D (**4**), the corresponding halogen derivatives (**3a**, **3b**, **4a**–**d**) and (7*S*)-stereoisomers (**5**–**8**, **7a**, **7b**, **8a**–**c**).

cell culture, have also been studied in this work.

2. Results and discussion

2.1. Chemistry

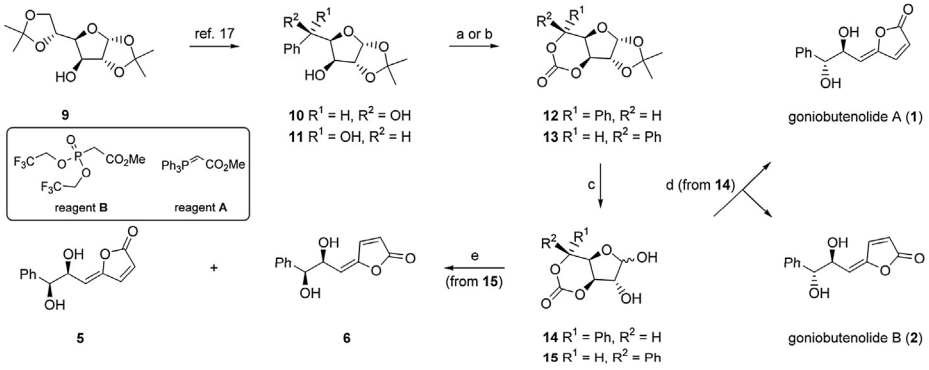
Synthesis of goniobutenolides A (**1**) and B (**2**) that started from the commercially available 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**9**) is shown in Scheme 1. Compound **9** was transformed into benzylic alcohols **10** and **11** by well established chemical reactions [17,18]. Cyclic carbonate can act as a protecting group [19] for the diol functionalities in **10** and **11**, but also it can undergo ring-opening reactions due to its good leaving group properties [20]. Treatment of **10** and **11** either with 1,1′-carbonyldiimidazole in toluene or with triphosgene and pyridine in dichloromethane afforded crystalline compounds **12** and **13** in high yields. Hydrolytic removal of the isopropylidene protective groups in both **12** and **13** gave the corresponding lactols **14** and **15** in almost quantitative yields. Both products are rather hygroscopic so they should be used in the next synthetic step immediately after their brief isolation.

In order to convert lactols **14** and **15** to the corresponding unsaturated lactones **1** and **2** we have studied two different olefination procedures, such as *Z*-selective Wittig reaction [5,21] with (methoxycarbonylmethylene)triphenylphosphorane (reagent A) and an alternative *Z*-selective olefination process in the presence of methyl *P*,*P*-bis(2,2,2-trifluoroethyl)phosphonoacetate (reagent B), under the standard Horner-Wadsworth-Emmons (HWE) conditions [22]. The stereoselectivity of both processes seems to strongly depend on the structure of the starting lactol, but also on the reaction temperature (for details, see Table S1 in the Supplementary data). The best combined yield of **1** and **2** (44%, *Z*/*E* ~2:1) was obtained when lactol **14** was allowed to react with Still's reagent (reagent **B**) under the HWE conditions (NaH, THF, Ar, 22 h, −10 °C). For possible mechanism of this process see Scheme S1 in the Supplementary data. Our optical rotation values were different from the values reported for synthetic [2–9] goniobutenolides A (1) and B (2) but were in reasonable agreement with the reported data of isolated **1** and **2** [1]. However, the NMR data of synthesized samples **1** and **2** were in full agreement with those reported in the literature for both isolated [1] and synthetic [2–9] samples **1** and **2**.

The most convenient procedure for the preparation of lactones **5** and **6** appeared to be a *Z*-selective Wittig reaction of **15** with (methoxycarbonylmethylene)triphenylphosphorane (reagent **A**), in anhydrous MeOH (Et_3N, 4 h, −10 °C). The desired unsaturated lactones **5** and **6** were thus obtained in 73% combined yield and in the approximate *Z*/*E* isomer ratio of 2:1.

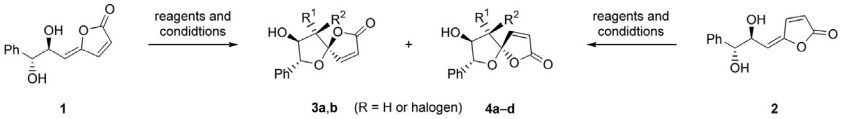
Stereochemistry of unsaturated lactones **2** and **5** were confirmed by single crystal X-ray diffraction analysis (for the crystal structures of **2** and **5**, see the Supplementary data).

Having obtained unsaturated lactones **1**, **2**, **5** and **6**, we next focused on the synthesis of 5-halogeno crassalactone D derivatives using different haloetherification protocols [23,24]. Iodoetherification of **2** with I_2 ($NaHCO_3$, CH_3CN) gave 5-iodo derivatives **3a** (14%), **4a** (27%) and **4b** (10%) (entry 2, Table 1). The same products were synthesized in slightly higher overall yield (**3a** 28%, **4a** 21%, **4b** 8%) after exposure of natural product **1** to the same reaction



Scheme 1. Reagents and conditions: (a) Imd_2CO, toluene, for **10**: reflux, 1.5 h, 95% of **12**, for **11**: 1.5 h, 80–85 °C, 76% of **13**; (b) triphosgene, Py, CH_2Cl_2, for **10**: 0 °C, 1 h, then rt, 1 h, 81% of **12**, for **11**: 0 °C, 1 h, then rt, 2 h, 83% of **13**; (c) 9:1 TFA/H_2O, 0 °C→rt, for **12**: 1.5 h, 100% of **14**, for **13**: 2 h, 96% of **15**; (d) reagent B, NaH, THF, Ar, 22 h, −10 °C, 29% of **1**, 15% of **2**; (e) reagent A, Et_3N, MeOH, N_2, 4 h, −10 °C, 49% of **5**, 24% of **6**.

Table 1
Haloetherification of goniobutenolides A (**1**) and B (**2**).



Entry	Starting compound	Reagents and conditions	Products (% yield)[a]
1	Goniobutenolide A (**1**)	I_2, $NaHCO_3$, CH_3CN, 6 days, rt→35 °C	**3a** R^1 = I, R^2 = H (28) **4a** R^1 = H, R^2 = I (21) **4b** R^1 = I, R^2 = H (8)
2	Goniobutenolide B (**2**)	I_2, $NaHCO_3$, CH_3CN, 7 days, rt→35 °C	**3a** R^1 = I, R^2 = H (14) **4a** R^1 = H, R^2 = I (27) **4b** R^1 = I, R^2 = H (10)
3	Goniobutenolide A (**1**)	NCS, DMF, 53 h, 35 °C	**4c** R^1 = Cl, R^2 = H (40)
4	Goniobutenolide B (**2**)	NCS, DMF, 70 h, 36 °C	**3b** R^1 = Cl, R^2 = H (26) **4d** R^1 = H, R^2 = Cl (17)

[a] Isolated yields.

conditions (entry 1, Table 1).

Treatment of **1** with NCS in DMF gave 5-chloro derivative **4c** in 40% (Table 1, entry 3). Under the similar conditions styryl-lactone **2** was transformed into the mixture of **3b** (26%) and **4d** (17%) (Table 1, entry 4).

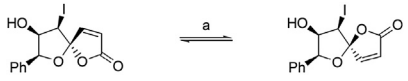
Iodoetherification of **5** and **6** (I_2, $NaHCO_3$) afforded 5-iodolactones **7b** and **8a** (9% and 40%, Table 2 entry 1; 3% and 51%, entry 2). Changing the source of iodine from I_2 to NIS gave the same products **7b** and **8a** but in lower yields (4% **7b** and 15% **8a** from **5**, entry 3; 12% **7b**, 19% **8a** from **6**, entry 4).

Chloroetherification of 7-epimers **5** and **6** afforded the same molecule **8b** in very low yields (13% from **5**, entry 5; 19% from **6**, entry 6).

Similarly, bromocyclisation of **5** (Br_2, $NaHCO_3$, CH_2Cl_2) gave compound **8c** in a yield of only 12% (entry 7). Bromoetherifaction of **5** was also attempted by using NBS in DMF. Under these reaction conditions product **8c** was obtained in a slightly better yield (29%, entry 8).

Stereoisomer **7a** could not be prepared by iodocyclisation of **5** or **6**. However treatment of **8a** with TFA in mixture of $CHCl_3/H_2O$ provided an equimolar mixture of **7a** and **8a**, thus providing a 42% yield of **7a** for biological testing (Scheme 2).

Stereochemistry of 5-halo-derivatives **3b**, **4c**,**d**, **7a**,**b** and **8a**–**c** were unequivocally confirmed by single crystal X-ray diffraction analysis (see Supplementary data for details). However, compounds



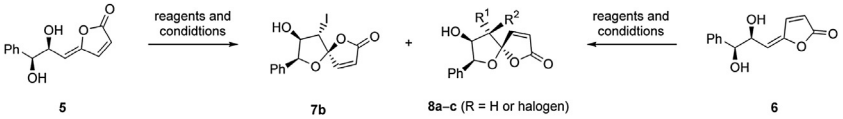
Scheme 2. Reagents and conditions: (a) 9:1 TFA/H_2O, $CHCl_3$, 5 days, 22 °C, 42% of **7a**, 42% of **8a**.

3a, **4a** and **4b** did not give the crystals of X-ray quality. In order to confirm the stereochemistry at C-4 of iodo derivative **3a**, it was dehalogenated with tributyltin hydride, in dry toluene and in the presence of AIBN, whereupon crassalactone D (**3**) was obtained in 81% yield (Scheme 3). This finding is consistent with (4*R*)-stereochemistry in **3a**. NOE interaction between H-5 and H-7 in **3a** confirmed its absolute configuration at C-5. Dehalogenation of both **4a** and **4b** under the same reaction conditions gave (4*R*)-crassalactone D (**4**) in 94% and 95% respective yields. These results are consistent with (4*S*)-stereochemistry of both **4a** and **4b**. NOE interactions between H-5 and Ph-H in **4a** and between H-5 and H-7 in **4b** confirmed their absolute configuration at C-5.

Dehalogenation of 5-iodo derivatives **7a** and **8a** under the reaction conditions similar to that described above provided the corresponding dehalogenated derivatives **7** (93%) and **8** (71%) as the only reaction products (Scheme 3).

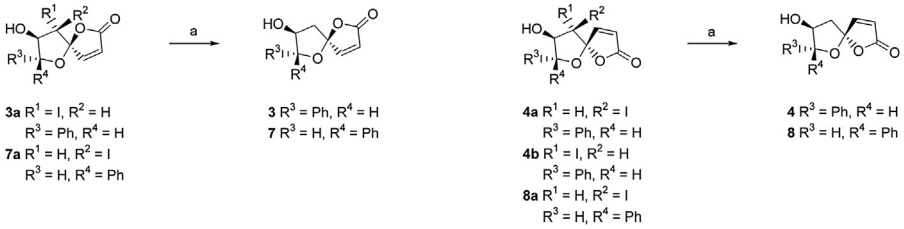
It appears, that the multi-step sequence comprised of the conversion of **10** to goniobutenolides **1** and **2**, followed by their

Table 2
Haloetherification of (7*S*)-goniobutenolides A (**5**) and B (**6**).



Entry	Starting compound	Reagents and conditions	Products (% yield)[a]
1	(7*S*)-goniobutenolide A (**5**)	I_2, $NaHCO_3$, CH_3CN, 9 days, rt→35 °C	**7b** (9) **8a** R^1 = H, R^2 = I (40)
2	(7*S*)-goniobutenolide B (**6**)	I_2, $NaHCO_3$, CH_3CN, 10 days, rt→35 °C	**7b** (3) **8a** R^1 = H, R^2 = I (51)
3	(7*S*)-goniobutenolide A (**5**)	NIS, CH_3CN, 48 h, rt	**7b** (4) **8a** R^1 = H, R^2 = I (15)
4	(7*S*)-goniobutenolide B (**6**)	NIS, DMF, 71 h, rt→50 °C	**7b** (12) **8a** R^1 = H, R^2 = I (19)
5	(7*S*)-goniobutenolide A (**5**)	NCS, DMF, 69.5 h, 40 °C	**8b** R^1 = Cl, R^2 = H (13)
6	(7*S*)-goniobutenolide B (**6**)	NCS, DMF, 69 h, 37 °C	**8b** R^1 = Cl, R^2 = H (19)
7	(7*S*)-goniobutenolide A (**5**)	Br_2, $NaHCO_3$, CH_2Cl_2, 2.5 h, rt	**8c** R^1 = Br, R^2 = H (12)
8	(7*S*)-goniobutenolide B (**6**)	NBS, DMF, atm. N_2, 20 days, rt→45°	**8c** R^1 = Br, R^2 = H (29)

[a] Isolated yields.



Scheme 3. Reagents and conditions: (a) Bu_3SnH, AIBN, toluene, 1 h, 100 °C, 81% of **3** (from **3a**), 94% of **4** (from **4a**), 95% of **4** (from **4b**), 93% of **7** (from **7a**), 71% of **8** (from **8a**).

subsequent iodoetherification (to **3a**, **3b**, **4a**–**d**), and their final free-radical dehalogenation (**3a**→**3**, **4a**,**b**→**4**) represent a new synthesis of (+)-crassalactone D (**3**) and (4*R*)-crassalactone D (**4**) from diacetone D-glucose (**9**) as a chiral precursor. By a similar reaction sequence compound **11** was converted to spiro-lactones **7** and **8** (via goniobutenolides **5** or **6** and iodo-lactones **7a**,**b** or **8a**–**c** as the key intermediates).

2.2. In vitro antitumour activities and SAR

Antiproliferative activities of synthesized natural products, their stereoisomers and analogues were evaluated against eight tumour cell lines (myelogenous leukaemia K562, promyelocytic leukaemia HL-60, Jurkat T cells leukaemia, Raji Burkitt's lymphoma, estrogen receptor positive breast adenocarcinoma MCF-7, estrogen receptor negative breast adenocarcinoma MDA-MB-231, cervix carcinoma HeLa, alveolar basal adenocarcinoma A549) and a single human normal cell line (MRC-5). The use of foetal lung fibroblasts (MRC-5) serves to demonstrate the toxicity of synthesized compounds toward normal cells. Cytotoxicity was evaluated by using the standard MTT assay, 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.

As shown in Table 3 the natural product **1** showed submicromolar activity toward promyelocytic leukaemia (HL-60), being notably more active than commercial cytostatic doxorubicin (DOX). Although somewhat less active than DOX, compound **1** acted as a very potent growth inhibitor of K562, MCF-7, HeLa, Jurkat and Raji cells (IC_{50} 0.35–1.99 μM). Unsaturated lactone **2** was found less active with respect to **1**. It demonstrated micromolar activities against the following cell lines: MCF-7, MDA-MB 231 and Hela, but these activities are significantly lower then those recorded for DOX in the same cell lines. (7*S*)-Goniobutenolide A (**5**) exhibited the most powerful activity against K562 cells being 1.7-fold more active than DOX and exhibited over 2-fold higher potency with respect to natural product **1**. Also, compound **5** showed submicromolar activity toward HeLa cells with a IC_{50} value 3-fold higher than that recorded for DOX against the same cell line. The highest potency in the culture of K562 cells between halogenated compounds was recorded after treatment with **4d** (IC_{50} 0.18 μM), which was 2-fold more potent than natural product **1**, 161-fold more potent than **2** and 1.4-fold more active than DOX. Other compounds showed micromolar activities (IC_{50} 1.21–11.68 μM). In the cultures of HL-60 and A549 cells the most active was 5-bromo derivative **8c** while other halogenated molecules showed moderate activities. A potent submicromolar activity was recorded after treatment of Jurkat cells with analogue **7a**, which was 2-fold more active than **1** and over 46-fold more potent than **2**. Iodolactone **7b** was 3-fold more active with respect to DOX, 106-fold more potent than **1** and approximately 2100-fold more active than **2**. Bromolactone **8c** showed the same antiproliferative activity as DOX (IC_{50} 0.03 μM) against Jurkat cells. However its activity was significantly stronger than that exhibited by both natural products **1** and **2** (35- and 700-fold respectively) in the same cell line. Several compounds exhibited very strong activities toward Raji cells. These are **4c** (IC_{50} 0.95 μM), **7a** (IC_{50} 0.07 μM), **7b** (IC_{50} 1.01 μM), **8c** (IC_{50} 2.37 μM) and their potencies are higher than that recorded for **1**, **2** and DOX. Derivatives **7a**, **7b** and **8c** also showed notable antiproliferative effects in the cultures of MCF-7 and MDA-MB-231 cells with IC_{50} values in the low submicromolar or micromolar range. Comparing to DOX, these analogues were more potent against MCF-7 cells but they showed the same or lower potency in the culture of MDA-MB 231 cells. All halogenated compound were moderately active toward HeLa cells. Remarkably, neither the natural products **1** and **2**,

Table 3
In vitro cytotoxicity of natural lactones **1** and **2**, their 7-epimers **5** and **6** halogenated analogues of crassalactone D (**3a**,**b**, **4a**–**c**, **7a**,**b**, **8a**–**c**) and DOX.

Compound	IC_{50} (μM)[a]								
	K562	HL-60	Jurkat	Raji	MCF-7	MDA-MB 231	HeLa	A549	MRC-5
1	0.35	0.25	1.06	3.55	1.08	>100	1.99	18.14	>100
2	28.94	45.78	21.01	32.74	4.74	8.11	5.34	>100	>100
5	0.15	33.54	32.32	12.22	13.53	2.89	0.21	74.23	>100
6	21.21	18.26	2.78	9.88	3.55	29.91	6.91	68.98	>100
3a	5.48	14.23	12.88	8.76	14.67	47.63	14.36	23.15	>100
3b	4.69	15.66	14.23	21.66	7.01	29.64	29.08	22.63	>100
4a	9.78	13.74	24.36	3.54	3.48	18.97	8.06	18.08	>100
4b	11.68	8.69	15.46	11.78	5.69	23.36	10.98	15.54	>100
4c	5.69	10.24	17.65	0.95	7.55	48.36	25.66	14.56	>100
4d	0.18	37.79	39.21	18.64	25.32	39.62	21.04	19.85	>100
7a	1.21	5.63	0.45	0.07	0.18	0.12	16.48	26.54	89.63
7b	6.78	24.79	0.01	1.01	0.01	1.32	6.69	21.85	91.21
8a	8.58	21.54	21.36	11.32	18.64	5.69	4.36	16.78	>100
8b	2.79	41.02	24.11	21.35	28.26	42.15	15.33	14.24	>100
8c	5.51	4.22	0.03	2.37	0.05	0.09	28.31	13.24	88.78
DOX	0.25	0.92	0.03	2.98	0.20	0.09	0.07	4.91	0.10

[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 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	3.87	35.54	40.22	20.37	62.42	3.93
2	3.01	32.11	44.23	20.65	3.99	3.87
5	2.57	40.34	39.02	18.07	4.10	3.46
6	1.57	44.03	36.25	18.15	0.75	3.87
3a	44.61	32.97	18.83	3.59	71.87	1.56
3b	20.05	18.21	49.2	12.54	78.38	3.66
4a	2.11	46.16	28.16	23.57	1.43	2.75
4b	24.02	24.51	40.37	11.1	84.50	3.49
4c	37.14	20.01	34.72	8.13	75.96	3.04
4d	51.01	24.49	21.45	3.06	69.61	3.50
7a	5.15	35.28	32.16	27.41	7.30	2.82
7b	8.25	45.27	29.13	17.35	9.45	2.16
8a	16.14	30.49	31.64	21.73	86.29	4.05
8b	2.58	44.01	31.20	22.21	0.76	2.02
8c	5.80	38.64	35.44	20.12	59.19	3.19

as well as their 7-epimers **5** and **6**, nor halogenated spiro-lactones were toxic toward normal human cells (MRC-5), while the commercial antitumour agent DOX exhibited sub-micromolar toxicity against this cell line (Table 4).

In order to establish possible structure-activity relationships we first considered the influence of double bond configuration to antitumour activity of unsaturated styryl lactones (see Fig. S11 in the Supplementary data for details). The importance of this structural feature for the cytotoxic activities was accessed by comparing the IC_{50} values of *Z*-isomers (**1**, **5**) and *E*-isomers (**2**, **6**). The results have shown that the geometry of double bond has different influence on antiproliferative activity of these molecules. Thus the presence of the *Z*-configured $C_4{=}C_5$ double bond in **1** significantly decreases the IC_{50} values, that is, increases the cytotoxicity of goniobutenolide A (**1**) with respect to goniobutenolide B (**2**) against seven of eight investigated cell lines. However, the same structural change in molecule **5** increases the IC_{50} values, namely decreases the cytotoxic activity of (7*S*)-goniobutenolide A (**5**) with respect to (7*S*)-goniobutenolide B (**6**) against five cell lines. It appears that the presence of *Z*-configured $C_4{=}C_5$ double bond increases the potency of the corresponding isomers against at least three malignant cell lines (K562, MDA-MB 231 and HeLa). Next, we studied the influence of absolute configuration at C-7 on growth-inhibitory activity of isomeric goniobutenolides. The data in Table 3 indicate that, in most cases, change of configuration from the 7*R* to the 7*S* increases antitumour activity. The same trend was observed when activities of 7*R* and 7*S* stereoisomeric halo-lactones were compared. Comparison of the IC_{50} values of 5*R* (**4a**, **4c**, **5a**) and 5*S* (**4b**, **4d**, **5b**) halogenated compounds revealed that molecules with 5*R* configuration were more cytotoxic. Finally, we considered influence of halogen atom type on antiproliferative activity of 5-halo-lactones. The results revealed that the analogues bearing iodine or bromine atom are more active than the corresponding 5-chloro derivatives. This suggests that the presence of a bulkier halogen atom increases antitumour activity of analogues.

2.3. Detection of apoptosis

Induction of apoptosis (a form of programmed cell death) in tumour cells is desirable mode of action for chemotherapeutic agents. Encouraged with findings that some of natural styryl-lactones from *Goniothalamus* genus induced apoptosis in various cell lines [25], we set the goal to examine apoptotic signalling induced by natural products **1** and **2**, their 7-epimers **5** and **6**, as well as by the halogenated spiro-lactones (**3a**,**b**, **4a**–**d**, **7a**,**b**, **8a**–**c**). In order to determine influence of synthesized compounds on cell distribution in cell cycle phases, K562 cells were treated with synthesized compounds in their IC_{50} concentration. Staining of apoptotic cells with propidium iodide, resulted in broad hypodiploid picks after flow cytometry analysis. Percentage of apoptotic, necrotic and live cells were determined with flow cytometry after double staining of treated cells with Annexin V-FITC/PI.

As data in Table 4 revealed all tested compounds increased percentage of K562 cells in sub G1 phase compared with untreated control. These findings suggested that synthesized lactones may induce apoptosis. Generally, both unsaturated and halogenated lactones with 7*R* configuration increased the cell percentage in sub G1 phase compared to stereoisomers of 7*S* configuration. Chloro derivatives **4d** and **4c** increased the cell percentage 34.5-fold and 25-fold respectively, while the iodo derivative **3a** induced 30-fold percentage of K562 cells in sub G1 phase compared with control. Analogues **3b**, **4b** and **8a** also significantly increase the percentages of K562 cells in sub G1 phase.

Although natural product **1** increases only 2.6-fold percentage of K562 cells in sub G1 phase with respect to control, it induced apoptosis in 62.42% of cells. Compounds **8a** was the most potent in inducing apoptosis, but generally compounds with 7*R* configuration induced apoptosis in larger number of cells compared with 7*S* isomeres. This is in agreement with the results obtained for the distribution of cells in the cell cycle phases. High percentage of specific apoptosis induced after treatment of K562 cells with lactones **4b** (84.50%), **3b** (78.38%), **3a** (71.87%), **4d** (69.61%) and **8c** (59.19%) is noteworthy. All tested compounds induced low percentage of specific necrosis in K562 cells (1.56–4.05%).

To resolve the mechanisms underlying the apoptosis induced with synthesized styryl lactones we have investigated the ability of these compounds (**1**, **2**, **5**, **6**, **3a**, **3b**, **4a**–**d**, **7a**, **7b**, **8a**–**c**) to modulate expression of some apoptosis proteins, such as Bcl-2, Bax, caspase 3 and PARP. Semi-quantitative Western blot analysis revealed that the most of examined compounds (**1**, **2**, **3a**, **3b**, **4a**, **4c**, **5**, **7a**, **8a**) reduced expression of anti-apoptotic Bcl-2 protein (cell guardians) in K562 cells compared with control (Fig. 2a). The majority of tested compounds (with the exception of **3a**, **3b**, **4a**, **6**) induced overexpression of pro-apoptotic effector protein Bax (Bcl-2 associated protein X, Fig. 2b) which when activated forms the oligomers that disrupt outer mitochondrial membrane. Cytochrome *c* released from damaged mitochondria promotes caspase 9 activation on the scaffold protein APAF1 [26,27].

Caspases, a family of aspartate-specific cysteine proteases, play central role in the mechanism of apoptosis as they both initiate and

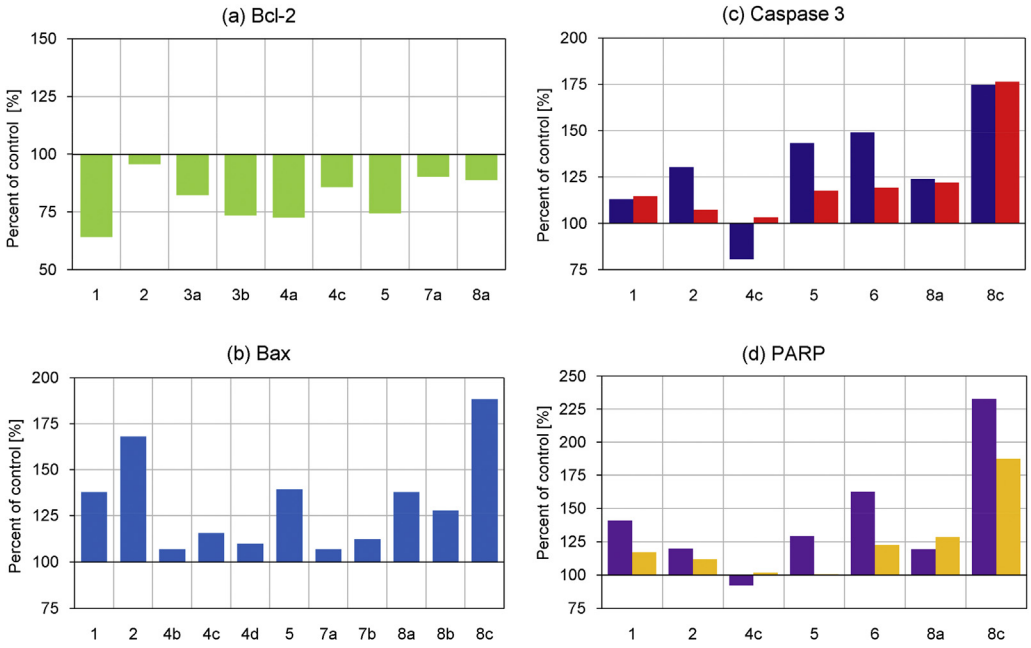


Fig. 2. Results of Western blot analysis after treatment of K562 cells with synthesized compounds.

execute the apoptotic process. Downstream, effector caspases (3, 6, 7) contribute to the biochemical and morphological changes that occur during the execution phase of apoptosis [28]. Three major pathways to apoptosis-associated caspase activation have been identified the extrinsic (death receptor), the intrinsic (mitochondrial) and the intrinsic endoplasmic reticulum pathway [29].

The expression level of the precursor and active subunit of effector caspase 3 and catalytic fragment of PARP in the cells exposed to synthesized compounds were measured in order to determine induced apoptosis associated with the activation of caspases. Caspase 3 activation is followed by cleavage of different downstream targets including poly ADP-ribose polymerase (PARP), which is specifically cleaved on 24 kDa DNA-binding domain and catalytic fragment (89 kDa) [30]. Western blot analysis showed cleavage of procaspase 3 (Fig. 2c) and PARP (Fig. 2d) in K562 cells after treatment with **1**, **2**, **4c**, **5**, **6**, **8a**, **8c**. Expression of activated caspase 3 higher than in control, but without cleavage of PARP, was also induced with **4a**, **4d** and **8b**. Induction of PARP cleavage without expression of activated caspase 3 was detected after treatment with halo-lactones **3b** and **7b** (see Fig. S18 in the Supplementary data).

Based on all this results we hypothesized that the most of investigated compounds induced apoptosis in K562 cells in caspase-dependent way. Biochemical changes in apoptosis, DNA fragmentation and caspase activation, may explain in part some of the morphological changes in apoptosis. Therefore, it is important to note that apoptosis may occur without oligonucleosomal DNA fragmentation and can be caspase-independent [31].

3. Conclusions

A new divergent synthesis of goniobutenolides A (**1**), B (**2**) and the corresponding 7-epimers has been achieved starting from diacetone D-glucose. The key step of the synthesis represents a novel cascade reaction comprised of a Z-selective Wittig (or HWE) olefination, followed by a concomitant lactonisation and the final E_2 elimination. HWE olefination afforded a higher yield of **1** and **2** (44%, *Z*/*E* ~ 2:1) compared with Wittig olefination. The highest yield and selectivity for **5** and **6** were achieved employing Wittig olefination at −10 °C (73%, *Z*/*E* ~ 2:1). Finally, unsaturated lactones **1**, **2**, **5** and **6** were converted to eleven novel halogenated crassalactone D derivatives (**3a**,**b**, **4a**–**d**, **7a**,**b**, **8a**–**c**) by using different haloetherification protocols.

Synthesized compounds were evaluated for their antiproliferative activity against a panel of malignant cell lines as well as against a single normal cell line by using the MTT test. Natural product **1** showed highest potency toward HL-60 cells (IC_{50} 0.25 μM). Some of the halogenated derivatives showed very potent antitumour activity, especially analogues **8c** (IC_{50} 0.03 μM against Jurkat cells) and **7a** (IC_{50} 0.07 μM toward Raji cells). 5-Iodo derivative **7b** was shown to be the most potent of all molecules under evaluation with IC_{50} 0.01 μM against Jurkat, and IC_{50} 0.01 μM against MCF-7 cells.

The preliminary SAR analysis suggested that: (a) the presence of the *Z*-configured double bond significantly decreases the IC_{50} values, that is, increases the cytotoxic activity of the corresponding goniobutenolides against at least three malignant cell lines; (b) changes in stereochemistry from 7*R* to 7*S* increases the antitumour activity both of goniobutenolides and of halo-lactones; (c) halo-lactones of 5*R* stereochemistry are more cytotoxic than the 5*S* isomers toward the majority of cells investigated; (d) the presence of 5-bromo and 5-iodo functionalities enhances antiproliferative activity compared to the activity of corresponding 5-chloro isosteres.

The flow-cytometry revealed that all synthesized compounds increase percentage of K562 cells in sub G1 phase compared with control, with analogue **4d** being the most potent (51.01% cells). High percentage (>50%) of specific apoptosis was detected for eight compounds (**1**, **3a**, **3b**, **4b**–**d**, **8a** and **8c**). Western blot analysis of apoptosis markers (Bcl-3, Bax, caspase 3, PARP) suggested that the majority of investigated compounds induced apoptosis in K562 cells in caspase-dependent way.

4. Experimental section

4.1. 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) of synthesized compounds were acquired on an Agilent technologies 6210 TOF LC/MS instrument (LC series 1200). 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 F254 as additive was used as stationary phase. All organic extracts were dried with anhydrous Na_2SO_4. Organic solutions were concentrated in a rotary evaporator under reduced pressure at a bath temperature below 30 °C. Purity of the tested compounds was determined by HRMS (errors were less than 5 ppm).

4.1.1. 3,5-O-Carbonyl-1,2-O-isopropyildene-5-C-phenyl-α-D-gluco-pentofuranose (**12**)

Procedure A: To a solution of **10** (0.962 g, 3.61 mmol) in dry toluene (40 mL) was added 1,1′-carbonyldiimidazole (1.464 g, 9.03 mmol) and the mixture was stirred at boiling temperature for 1.5 h. After the mixture was cooled down to room temperature it was concentrated and the residue purified by flesh chromatography (4:1 light petroleum/EtOAc) to afford pure **12** (0.998 g, 95%).

Procedure B: To a cooled (0 °C) solution of **10** (0.323 g, 1.21 mmol) in mixture of dry pyridine (0.71 mL, 8.83 mmol) and dry CH_2Cl_2 (9.2 mL) solution of triphosgene (0.215 g, 0.73 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise. The mixture was stirred at 0 °C for 1 h and then at room temperature for 1 h. The mixture was poured into saturated solution of NH_4Cl and extracted first with CH_2Cl_2 (2 × 15 mL) and then with Et_2O (3 × 15 mL). The combined organic solutions were washed with brine, dried and evaporated. The residue was purified by flash column chromatography (9:1 Et_2O/light petroleum) to afford pure **12** (0.285 g, 81%). Recrystallization from CH_2Cl_2/hexane gave white needles, mp 138 °C, $[\alpha]_D = +33.2$ (*c* 1, $CHCl_3$), $R_f = 0.25$ (4:1 light petroleum/EtOAc). IR (film): ν_{max} 1764 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.29 and 1.45 (2 × s, 3H each, C**Me**$_2$), 4.50 (dd, 1H, $J_{3,4} = 2.4$, $J_{4,5} = 2.2$ Hz, H-4), 4.68 (d, 1H, $J_{3,4} = 2.7$ Hz, H-3), 4.72 (d, 1H, $J_{1,2} = 3.6$ Hz, H-2), 5.73 (bs, 1H, H-5), 6.02 (d, 1H, $J_{1,2} = 3.6$ Hz, H-1), 7.23–7.5 (m, 5H, Ph). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 25.8 and 26.3 (C**Me**$_2$), 73.8 (C-4), 79.4 (C-5), 79.8 (C-3), 83.1 (C-2), 104.8 (C-1), 112.8 (**C**Me$_2$), 124.8, 129.0, 129.1, 134.9 (Ph), 146.0 (C=O). HRMS (ESI): *m/e* 293.1009 (M^++H), calcd for $C_{15}H_{17}O_6$: 293.1020; *m/e* 331.0566 (M^++K), calcd for $C_{15}H_{16}KO_6$: 331.0578.

4.1.2. 3,5-O-Carbonyl-1,2-O-isopropyildene-5-C-phenyl-β-L-ido-pentofuranose (**13**)

Procedure A: To a solution of **11** (0.210 g, 0.79 mmol) in dry toluene (4 mL) was added 1,1′-carbonyldiimidazole (0.192 g, 1.18 mmol) and the mixture was stirred at 80–85 °C for 1.5 h. After the mixture was cooled down to room temperature it was concentrated and the residue purified by flesh column chromatography (24:1 CH_2Cl_2/Me_2CO) to afford pure **13** (0.174 g, 76%).

Procedure B: To a cooled (0 °C) solution of **11** (0.394 g, 1.47 mmol) in a mixture of dry pyridine (0.12 mL) and CH_2Cl_2 (7 mL) a solution of triphosgene (0.144 g, 0.48 mmol) in dry CH_2Cl_2 (0.3 mL) was added dropwise. The mixture was stirred at 0 °C for 1 h and then at room temperature for 1 h. A new portion of dry pyridine (0.05 mL) and solution of triphosgene (0.087 g, 0.16 mmol) in dry CH_2Cl_2 (0.2 mL) was added and stirring at room temperature was continued for additional 1 h. The mixture was poured into saturated solution of NH_4Cl and extracted first with CH_2Cl_2 (2 × 7 mL) and then with Et_2O (3 × 7 mL). The combined organic solutions were washed with brine, dried and evaporated. The residue was purified by flash column chromatography (24:1 CH_2Cl_2/Me_2CO) to afford pure **13** (0.432 g, 83%). Recrystallization from CH_2Cl_2/hexane gave short white needles, mp 187 °C, $[\alpha]_D = +23.8$ (*c* 0.5, $CHCl_3$), $R_f = 0.45$ (49:1 CH_2Cl_2/Me_2CO). IR (film): ν_{max} 1761 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.32 and 1.46 (2 × s, 3H each, C**Me**$_2$), 4.65 (dd, 1H, $J_{3,4} = 3.0$, $J_{4,5} = 1.2$ Hz, H-4), 4.78 (d, 1H, $J_{1,2} = 3.7$ Hz, H-2), 5.01 (d, 1H, $J_{3,4} = 3.1$ Hz, H-3), 5.55 (bs, 1H, H-5), 6.01 (d, 1H, $J_{1,2} = 3.7$ Hz, H-1), 7.35–7.51 (m, 5H, Ph). NOE contact H-1 and Ph-H, H-2 and Ph-H. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 26.1 and 26.5 (C**Me**$_2$), 72.6 (C-4), 77.7 (C-5), 82.8 (C-3), 83.1 (C-2), 105.1 (C-1), 112.9 (**C**Me$_2$), 126.9, 128.5, 129.1, 134.0 (Ph), 146.8 (C=O). HRMS (ESI): *m/e* 293.1005 (M^++H), calcd for $C_{15}H_{17}O_6$: 293.1020; *m/e* 331.0564 (M^++K), calcd for $C_{15}H_{16}KO_6$: 331.0578.

4.1.3. (+)-goniobutenolide A (**1**) and (−)-goniobutenolide B (**2**)

Solution of **12** (0.364 g, 1.24 mmol) in 90% TFA (5 mL) was stirred for 30 min 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 **14** (0.347 g, 100%), $[\alpha]_D = -73.8$ (*c* 1.0, EtOH), $R_f = 0.26$ (24:1 CH_2Cl_2/MeOH). IR (KBr): ν_{max} 3411 (OH), 1786 (C = O). HRMS (ESI): *m/e* 297.0602 (M + $HCOO^-$), calcd for $C_{13}H_{13}O_8$: 297.0616. To a cooled (−10 °C) and stirred suspension of 95% NaH (0.036 g, 1.51 mmol) in dry THF (4 mL) was added dropwise [bis-(2,2,2-trifluoro-ethoxy)-phosphoryl]-acetic acid methyl ester (reagent **B**; 0.3 mL, 1.45 mmol). The resulting mixture was stirred in argon atmosphere for 0.5 h and a solution of **14** (0.190 g, 0.75 mmol) in dry THF (4 mL) was added. After being stirred at −10 °C in argon atmosphere for 22 h the mixture was poured into 5% aq HCl (20 mL) and the resulting suspension was extracted first with Et_2O (3 × 10 mL) and then with EtOAc (3 × 10 mL). Combined organic solutions were washed with brine, dried and concentrated. Purification by flash column chromatography (4:1 Et_2O/light petroleum) followed by preparative TLC (19:1 $^iPr_2O$/MeOH, three successive developments for **1** and **2**) afforded pure **1** (0.052 g, 29%) and **2** (0.026 g, 15%).

*4.1.3.1. (+)-goniobutenolide A (**1**).* Colourless syrup, $[\alpha]_D = +86.8$ (*c* 0.4, $CHCl_3$), $R_f = 0.28$ (19:1 $^iPr_2O$/MeOH), lit [1]. $[\alpha]_D = +82.0$ (*c* 0.25, $CHCl_3$). Both ^{1}H and ^{13}C NMR data of compound **1** are consistent with the naturally occurring goniobutenolide A [1], and are in good agreement with the data previously reported by us [16].

*4.1.3.2. (−)-goniobutenolide B (**2**).* Colourless sticks, mp 142–145 °C (CH_2Cl_2), $[\alpha]_D = -39.0$ (*c* 0.1, $CHCl_3$), $R_f = 0.33$ (19:1 DIPE/MeOH), lit [1]. mp 148–149 °C, $[\alpha]_D = -36.5$ (c 0.2, $CHCl_3$). ^{1}H and ^{13}C NMR data of compound **2** are consistent with the naturally occurring goniobutenolide B [1], and are in good agreement with the data previously reported by us [16].

4.1.4. (7S)-goniobutenolide A (**5**) and (7S)-goniobutenolide B (**6**)

Solution of **13** (0.367 g, 1.24 mmol) in 90% TFA (10.5 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 resulting residue was purified by flash column chromatography (47:3 → 23:2 CH_2Cl_2/MeOH) to afford pure lactol **15** (0.302 g, 96%) as a colourless syrup. $[\alpha]_D = +29.0$ (*c* 1.0, EtOH), $R_f = 0.53$ (23:2 CH_2Cl_2/MeOH). IR (CH_2Cl_2): ν_{max} 3434 (OH), 1786–1746 (C=O). HRMS (ESI): *m/e* 253.0715 (M^++H), calcd for $C_{12}H_{13}O_6$: 253.0707; *m/e* 270.0984 (M^++NH_4), calcd for $C_{12}H_{16}NO_6$: 270.0972; *m/e* 291.0280 (M^++K), calcd for $C_{12}H_{12}KO_6$: 291.0266. To a cooled (−10 °C) and stirred solution of **15** (0.186 g, 0.74 mmol) in dry MeOH (8 mL) were added three equal portions on every 40 min

of Et_3N (0.06 mL, 0.44 mmol) and 2-(triphenylphosphoranylidene)-acetic acid methyl ester (reagent **A**, 0.148 g, 0.44 mmol). The mixture was stirred under nitrogen atmosphere at −10 °C for 4 h and then evaporated. Multiple purification by flesh column chromatography (9:1 Et_2O/light petroleum) followed by preparative TLC (4:1 CH_2Cl_2/EtOAc, three successive developments for **5** and **6**) afford pure **5** (0.084 g, 49%) and **6** (0.041 g, 24%).

*4.1.4.1. (7S)-goniobutenolide A (**5**).* Colourless needles, mp 94–95 °C (EtOAc/hexane, at 0 °C), $[\alpha]_D = +110.7$ (*c* 0.3, $CHCl_3$), $R_f = 0.29$ (19:1 $^iPr_2O$/MeOH), lit [4]. mp 94–96 °C, $[\alpha]_D = +144.0$ (*c* 0.3, $CHCl_3$). ^1H and ^{13}C NMR data for **5** matched those previously reported in the literature [4,16].

*4.1.4.2. (7S)-Goniotebutenolide B (**6**).* Colourless needles, mp 115–118 °C (CH_2Cl_2), $[\alpha]_D = -134.3$ (*c* 0.3, $CHCl_3$), $R_f = 0.39$ (19:1 $^iPr_2O$/MeOH), lit [4]. mp 117–119 °C, $[\alpha]_D = -196.0$ (*c* 0.3, $CHCl_3$). ^1H and ^{13}C NMR data for **5** matched those previously reported in the literature [4,16].

*4.1.5. (4R,5S,7R)-5-iodo-crassalactone D (**3a**), (4S,5R,7R)-5-iodo-crassalactone D (**4a**) and (4S,5S,7R)-5-iodo-crassalactone D (**4b**)*

Procedure A: Solution of **1** (0.091 g, 0.39 mmol), I_2 (0.329 g, 1.3 mmol) and $NaHCO_3$ (0.109 g, 1.3 mmol) in dry CH_3CN (5.5 mL) was stirred at room temperature for 0.5 h and then at 35 °C for 6 days. Mixture was poured in H_2O (25 mL) and resulting suspension was extracted with CH_2Cl_2 (3 × 15 mL). Combined organic solutions were washed with 10% sodium sulphite (25 mL) and brine (25 mL), dried and concentrated. The residue was purified by preparative TLC (1:1 Et_2O/light petroleum, two successive developments) to afford impure **4b** and a mixture of **3a** and **4a** which was separated by preparative TLC (49:1 CH_2Cl_2/Me_2CO, two successive developments). Pure **3a** (0.04 g, 28%) and **4a** (0.029 g, 21%) were isolated. Purification of impure **4b** by preparative TLC (1:1 Et_2O/light petroleum, two successive developments) afforded pure **4b** (0.012 g, 8%).

Procedure B: Suspension of **2** (0.036 g, 0.16 mmol), I_2 (0.135 g, 0.53 mmol) and $NaHCO_3$ (0.043 g, 0.53 mmol) in dry CH_3CN (2.5 mL) was stirred at room temperature for 0.5 h and then at 35 °C for seven days. The mixture was poured in H_2O (20 mL) and resulting suspension was extracted with CH_2Cl_2 (3 × 10 mL). Combined organic solutions were washed with 10% sodium sulphite (20 mL) and brine (20 mL), dried and concentrated. The residue was purified by preparative TLC (1:1 Et_2O/light petroleum) to afford pure **4b** (0.006 g, 10%) and a mixture of **3a** and **4a** which was separated by preparative TLC (49:1 CH_2Cl_2/Me_2CO, two successive developments). Pure **3a** (0.008 g, 14%) and **4a** (0.015 g, 27%) were isolated.

*4.1.5.1. (4R,5S,7R)-5-iodo-crassalactone D (**3a**).* Colourless syrup, $[\alpha]_D = +26.0$ (*c* 0.2, $CHCl_3$), $R_f = 0.49$ (49:1 CH_2Cl_2/Me_2CO), IR (film): ν_{max} 3445 (OH), 1779 (C=O). ^1H NMR (250 MHz, $CDCl_3$): δ 3.20 (bs, 1H, OH), 4.45–4.63 (m, 2H, H-5 and H-6), 5.10 (d, 1H, $J_{6,7} = 5.5$ Hz, H-7), 6.20 (d, 1H, $J_{2,3} = 5.5$ Hz, H-2), 7.30–7.44 (m, 6H, H-3 and Ph). NOE contact: H-5 and. H-7. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 28.6 (C-5), 86.4 (C-6), 87.6 (C-7), 113.1 (C-4), 124.0 (C-2). 125.9, 128.6, 128.7, 136.7 (Ph), 153.6 (C-3), 169.4 (C-1). HRMS (ESI): *m/e* 376.0040 (M^++NH_4), calcd for $C_{13}H_{15}NIO_4$: 376.0040.

*4.1.5.2. (4S,5R,7R)-5-iodo-crassalactone D (**4a**).* Colourless syrup, $[\alpha]_D = -5.5$ (*c* 0.2, $CHCl_3$), $R_f = 0.61$ (49:1 CH_2Cl_2/Me_2CO). IR (film): ν_{max} 3459 (OH), 1770 (C=O). ^1H NMR (250 MHz, $CDCl_3$): δ 2.74 (bs, 1H, OH), 3.91 (m, 1H, H-6), 4.67 (d, 1H, $J_{5,6} = 3.6$ Hz, H-5), 5.10 (d, 1H, $J_{6,7} = 7.1$ Hz, H-7), 6.22 (d, 1H, $J_{2,3} = 5.5$ Hz, H-2), 7.30–7.56 (m, 5H, Ph), 7.53 (d, 1H, $J_{2,3} = 5.6$ Hz, H-3). NOE contact: H-5 and Ph-H. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 37.7 (C-5), 76.8 (C-6), 86.7 (C-7), 113.7 (C-4), 124.9 (C-2), 126.3, 128.7, 128.8, 137.1 (Ph), 154.3 (C-3), 169.1 (C-1). HRMS (ESI): *m/e* 376.0041 (M^++NH_4), calcd for $C_{13}H_{15}NIO_4$: 376.0040.

*4.1.5.3. (4S,5S,7R)-5-iodo-crassalactone D (**4b**).* Colourless syrup, $[\alpha]_D = -32.5$ (*c* 0.2, $CHCl_3$), $R_f = 0.70$ ($^iPr_2O$). IR (film): ν_{max} 3445 (OH), 1770 (C=O). ^1H NMR (250 MHz, $CDCl_3$): δ 2.82 (bs, 1H, OH), 4.49 (d, 1H, $J_{5,6} = 10.1$ Hz, H-5), 4.57 (dd, 1H, $J_{5,6} = 10.2$, $J_{6,7} = 7.4$ Hz, H-6), 4.95 (d, 1H, $J_{6,7} = 7.4$ Hz, H-7), 6.37 (d, 1H, $J_{2,3} = 5.6$ Hz, H-2), 7.09 (d, 1H, $J_{2,3} = 5.6$ Hz, H-3), 7.30–7.54 (m, 5H, Ph). NOE contact: H-5 and H-7, H-3 and H-5. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 31.3 (C-5), 83.7 (C-6), 86.4 (C-7), 111.1 (C-4), 126.4, 127.2, 128.77, 128.8, 137.8 (C-2 and Ph), 149.1 (C-3), 168.6 (C-1). HRMS (ESI): *m/e* 376.0041 (M^++NH_4), calcd for $C_{13}H_{15}NIO_4$: 376.0040.

*4.1.6. (4R,5S,7S)-5-iodo-crassalactone D (**7b**) and (4S,5R,7S)-5-iodo-crassalactone D (**8a**)*

Procedure A: Solution of **5** (0.109 g, 0.47 mmol), I_2 (0.412 g, 1.64 mmol) and $NaHCO_3$ (0.138 g, 1.64 mmol) in dry CH_3CN (6.5 mL) was stirred at room temperature for 0.5 h and then at 35 °C for 9 days. The mixture was poured in H_2O (20 mL) and resulting suspension was extracted with CH_2Cl_2 (4 × 10 mL). Combined organic solutions were washed with 10% sodium sulphite (20 mL) and brine (20 mL), dried and concentrated. The residue was purified by preparative TLC (1:1 Et_2O/light petroleum, two successive developments) to afford pure **8a** (0.068 g, 40%) and impure **7b** which was additionally purified by preparative TLC ($^iPr_2O$, two successive developments). Pure **7b** was isolated (0.0144 g, 9%) as a colourless solid.

Procedure B: Solution of **5** (0.044 g, 0.19 mmol) and NIS (0.064 g, 0.28 mmol) in dry CH_3CN (1.2 mL) was stirred at room temperature for 48 h. The mixture was poured into 10% sodium sulphite (20 mL) and resulting suspension was extracted with CH_2Cl_2 (3 × 10 mL). Combined organic solutions were washed with brine (20 mL), dried and concentrated. The residue was purified by preparative TLC ($^iPr_2O$) to afford pure **8a** (0.010 g, 15%) and **7b** (0.003 g, 4%).

Procedure C: Solution of **6** (0.022 g, 0.09 mmol), I_2 (0.078 g, 0.31 mmol) and $NaHCO_3$ (0.026 g, 0.31 mmol) in dry CH_3CN (1.9 mL) was stirred at room temperature for 0.5 h and then at 35 °C for ten days. Mixture was poured in H_2O (20 mL) and the resulting suspension was extracted with CH_2Cl_2 (4 × 10 mL). Combined organic solutions were washed with 10% sodium sulphite (20 mL) and brine (20 mL), dried and concentrated. The residue was purified by preparative TLC (1:1 Et_2O/light petroleum, two successive developments) to afford pure **8a** (0.017 g, 51%) and impure **7b** which was additionally purified by preparative TLC ($^iPr_2O$, two successive developments). Pure **7b** was isolated (0.001 g, 3%) as a colourless solid.

Procedure D: Solution of **6** (0.031 g, 0.14 mmol) and NIS (0.061 g, 0.27 mmol) in dry DMF (2 mL) was stirred at room temperature for 0.5 h and then at 40 °C additional 46.5 h. A new portion of NIS (0.031 g, 0.14 mmol) was added and stirring continued for 24 h at 50 °C. The mixture was diluted with EtOAc (5 mL). Organic solution was washed with 10% sodium sulphite (20 mL) and brine (20 mL), dried and concentrated. The residue was purified by preparative TLC (7:3 Et_2O/light petroleum) to give pure **8a** (0.009 g, 19%) and **7b** (0.006 g, 12%).

*4.1.6.1. (4R,5S,7S)-5-iodo-crassalactone D (**7b**).* Colourless needles, mp 108–112 °C (Et_2O/hexane), $[\alpha]_D = +12.0$ (*c* 0.2, $CHCl_3$), $R_f = 0.56$ ($^iPr_2O$). IR (KBr): ν_{max} 3464 (OH), 1777 (C=O). ^1H NMR (250 MHz, $CDCl_3$): δ 2.47 (d, 1H, $J_{6,OH} = 9.9$ Hz, OH), 4.54 (s, 1H, H-5), 4.72 (dd, 1H, $J_{6,7} = 4.2$, $J_{6,OH} = 9.6$ Hz, H-6), 5.99 (d, 1H, $J_{6,7} = 4.3$ Hz, H-7), 6.28 (d, 1H, $J_{2,3} = 5.6$ Hz, H-2), 7.31–7.50 (m, 5H, Ph), 7.55 (d, 1H,

$J_{2,3}$ = 5.7 Hz, H-3). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 29.8 (C-5), 81.3 (C-6), 86.1 (C-7), 114.8 (C-4), 125.5 (C-2), 126.5, 128.6, 128.8, 134.4 (Ph), 154.0 (C-3), 168.4 (C-1). HRMS (ESI): *m/e* 376.0041 (M^++NH_4), calcd for $C_{13}H_{15}NIO_4$: 376.0040.

*4.1.6.2. (4S,5R,7S)-5-iodo-crassalactone D (**8a**).* Colourless needles, mp 107–110 °C (CH_2Cl_2/hexane), $[\alpha]_D$ = −9.0 (*c* 0.2, $CHCl_3$), R_f = 0.42 (1:1 Et_2O/light petroleum). IR (film): ν_{max} 3404 (OH), 1760 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.92 (bs, 1H, OH), 4.41 (t, 1H, $J_{5,6}$ = 3.5 Hz, $J_{6,7}$ = 3.2 Hz, H-6), 4.94 (d, 1H, $J_{5,6}$ = 3.9 Hz, H-5), 5.56 (d, 1H, $J_{6,7}$ = 2.8 Hz, H-7), 6.12 (d, 1H, $J_{2,3}$ = 5.5 Hz, H-2), 7.33–7.59 (m, 6H, Ph and H-3). NOE contact: H-5 and H-7. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 30.2 (C-5), 74.9 (C-6), 83.8 (C-7), 114.2 (C-4), 122.2 (C-2), 126.6, 128.7, 128.9, 134.2 (Ph), 154.8 (C-3), 169.8 (C-1). HRMS (ESI): *m/e* 376.0029 (M^++NH_4), calcd for $C_{13}H_{15}NIO_4$: 376.0040.

*4.1.7. (4R,5R,7S)-5-iodo-crassalactone D (**7a**)*

To a stirred solution of **8a** (0.031 g, 0.09 mmol) in a mixture of $CHCl_3$ (1.5 mL) and H_2O (0.01 mL) was added TFA (0.06 mL, 0.86 mmol). The resulting mixture was stirred at 22 °C for 5 days and then concentrated by co-distillation with toluene. The resulting residue was purified by preparative TLC (7:3 Et_2O/light petroleum) to give pure **7a** (0.013 g, 42%) and **8a** (0.013 g, 42%). Recrystallization from CH_2Cl_2/hexane gave pure **7a** as long colourless prisms, mp 163–167 °C (CH_2Cl_2/hexane), $[\alpha]_D$ = +19.0 (*c* 0.1, $CHCl_3$), R_f = 0.58 (4:1 Et_2O/light petroleum). IR (KBr): ν_{max} 3461 (OH), 1779–1739 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.20 (bs, 1H, OH), 4.45 (bs, 1H, H-6), 4.67 (d, 1H, $J_{5,6}$ = 4.4 Hz, H-5), 5.50 (d, 1H, $J_{6,7}$ = 4.1 Hz, H-7), 6.38 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-2), 7.09 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-3), 7.31–7.57 (m, 5H, Ph). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 30.9 (C-5), 73.7 (C-6), 86.3 (C-7), 112.5 (C-4), 126.7, 127.0, 128.55, 128.6, 134.8 (C-2 and Ph), 149.2 (C-3), 168.4 (C-1). HRMS (ESI): *m/e* 376.0040 (M^++NH_4), calcd for $C_{13}H_{15}NIO_4$: 376.0040.

*4.1.8. (4S,5S,7R)-5-Chloro-crassalactone D (**4c**)*

Solution of **1** (0.050 g, 0.21 mmol) and NCS (0.043 g, 0.32 mmol) in dry DMF (1.3 mL) was stirred at 35 °C for 53 h. The mixture was diluted with EtOAc (7 mL). Organic solution was washed with 10% sodium sulphite (20 mL) and brine (20 mL), dried and concentrated. The residue purification by preparative TLC (^{i}Pr_2O) afforded pure **4c** (0.023 g, 40%). Recrystallization from CH_2Cl_2/hexane gave pure **4c** as colourless plates, mp 130–134 °C (CH_2Cl_2/hexane), $[\alpha]_D$ = +32.3 (*c* 0.3, $CHCl_3$), R_f = 0.45 (^{i}Pr_2O). IR (KBr): ν_{max} 3423 (OH), 1745 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 3.10 (d, 1H, $J_{6,OH}$ = 3.4 Hz, OH), 4.37 (d, 1H, $J_{5,6}$ = 9.5 Hz, H-5), 4.47 (m, 1H, $J_{6,7}$ = 7.7, $J_{5,6}$ = 9.2, $J_{6,OH}$ = 3.4 Hz, H-6), 4.95 (d, 1H, $J_{6,7}$ = 7.6 Hz, H-7), 6.35 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-2), 7.14 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-3), 7.29–7.53 (m, 5H, Ph). NOE contact: H-3 and H-5. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 63.5 (C-5), 81.6 (C-6), 84.8 (C-7), 110.4 (C-4), 126.9 (C-2), 126.4, 128.8, 128.9, 137.7 (Ph), 148.8 (C-3), 168.7 (C-1). HRMS (ESI): *m/e* 284.0696 (M^++NH_4), calcd for $C_{13}H_{15}NClO_4$: 284.0684.

*4.1.9. (4R,5S,7R)-5-Chloro-crassalactone D (**3b**) and (4S,5R,7R)-5-chloro-crassalactone D (**4d**)*

Solution of **2** (0.010 g, 0.43 mmol) and NCS (0.086 g, 0.64 mmol) in dry DMF (2 mL) was stirred at 36 °C for 70 h. The mixture was diluted with EtOAc (5 mL). Organic solution was washed with 10% sodium sulphite (20 mL) and brine (20 mL), dried and concentrated. Multiple purification by flesh chromatography (1:1 Et_2O/light petroleum) afforded pure **3b** (0.029 g, 26%) and **4d** (0.019 g, 17%).

*4.1.9.1. (4R,5S,7R)-5-Chloro-crassalactone D (**3b**).* Colourless needles, mp 106–109 °C (CH_2Cl_2/hexane), $[\alpha]_D$ = +11.3 (*c* 0.3, $CHCl_3$), R_f = 0.49 (^{i}Pr_2O). IR (KBr): ν_{max} 3430 (OH), 1765 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.88 (bs, 1H, OH), 4.39–4.52 (m, 2H, H-5 and H-6), 5.15 (d, 1H, $J_{6,7}$ = 4.8 Hz, H-7), 6.35 (d, 1H, $J_{2,3}$ = 5.7 Hz, H-2), 7.32–7.50 (m, 6H, H-3, Ph). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 66.2 (C-5), 85.2 (C-6), 88.9 (C-7), 113.6 (C-4), 125.4 (C-2), 126.0, 128.7, 128.8, 137.0 (Ph), 149.8 (C-3), 168.5 (C-1). HRMS (ESI): *m/e* 284.0687 (M^++NH_4), calcd for $C_{13}H_{15}NClO_4$: 284.0684.

*4.1.9.2. (4S,5R,7R)-5-Chloro-crassalactone D (**4d**).* Colourless needles, mp 110–111 °C (CH_2Cl_2/hexane), $[\alpha]_D$ = −1.0 (*c* 0.2, $CHCl_3$), R_f = 0.46 (^{i}Pr_2O). IR (KBr): ν_{max} 3446 (OH), 1766 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 2.53 (d, 1H, $J_{6,OH}$ = 8.4 Hz, OH), 4.52 (d, 1H, $J_{5,6}$ = 4.4 Hz, H-5), 4.70 (m, 1H, H-6), 5.09 (d, 1H, $J_{6,7}$ = 7.8 Hz, H-7), 6.34 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-2), 7.33–7.51 (m, 5H, Ph), 7.48 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-3). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 66.1 (C-5), 77.2 (C-6), 85.7 (C-7), 112.9 (C-4), 125.5 (C-2), 126.3, 126.4, 128.8, 137.2 (Ph), 150.6 (C-3), 168.5 (C-1). HRMS (ESI): *m/e* 284.0684 (M^++NH_4), calcd for $C_{13}H_{15}NClO_4$: 284.0684.

*4.1.10. (4S,5S,7S)-5-Chloro-crassalactone D (**8b**)*

Procedure A: Solution of **5** (0.037 g, 0.16 mmol) and NCS (0.042 g, 0.32 mmol) in dry DMF (1.1 mL) was stirred at 40 °C for 69.5 h. The mixture was diluted with EtOAc (7 mL) and poured in 10% sodium sulphite (20 mL). Organic layer was separated and water solution extracted with EtOAc (3 × 9 mL). Combined organic solution were washed with brine (20 mL), dried and concentrated. Double purification by TLC (^{i}Pr_2O, two successive developments) afforded pure **8b** (0.006 g, 13%).

Procedure B: Solution of **6** (0.035 g, 0.15 mmol) and NCS (0.040 g, 0.30 mmol) in dry DMF (0.85 mL) was stirred at 37 °C for 69 h. The mixture was diluted with EtOAc (5 mL) and poured in 10% sodium sulphite (20 mL). Organic layer was separated and water solution extracted with EtOAc (3 × 9 mL). Combined organic solution were washed with brine (20 mL), dried and concentrated. Purification by preparative TLC (^{i}Pr_2O) afforded pure **8b** (0.008 g, 19%), as colourless irregular shaped crystals, mp 145–148 °C (CH_2Cl_2/hexane), $[\alpha]_D$ = −33.0 (*c* 0.2, $CHCl_3$), R_f = 0.43 (^{i}Pr_2O). IR (KBr): ν_{max} 3456 (OH), 1772 (C=O). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.78 (br s, 1H, OH), 4.32 (d, 1H, $J_{5,6}$ = 6.6 Hz, H-5), 4.83 (t, 1H, $J_{5,6} \approx J_{6,7}$ = 6.8 Hz, H-6), 5.62 (d, 1H, $J_{6,7}$ = 6.9 Hz, H-7), 6.38 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-2), 7.30 (d, 1H, $J_{2,3}$ = 5.6 Hz, H-3), 7.31–7.59 (m, 5H, Ph). NOE contact: H-3 and H-5. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 63.9 (C-5), 77.8 (C-6), 83.0 (C-7), 110.0 (C-4), 126.5 (C-2), 127.1, 128.8, 129.1, 134.0 (Ph), 148.8 (C-3), 173.5 (C-1). HRMS (ESI): *m/e* 284.0683 (M^++NH_4), calcd for $C_{13}H_{15}NClO_4$: 284.0684.

*4.1.11. (4S,5S,7S)-5-bromo-crassalctone D (**8c**)*

Procedure A: Suspension of **5** (0.042 g, 0.18 mmol), Br_2 (0.2 mL, 0.36 mmol) and $NaHCO_3$ (0.02 g, 0.36 mmol) in dry CH_2Cl_2 (2 mL) was stirred at room temperature for 2.5 h. The mixture was poured in 10% sodium sulphite and extracted with CH_2Cl_2 (4 × 10 mL). Organic solution was washed with water (20 mL) and brine (20 mL), dried and concentrated. The residue was purified by preparative TLC (7:3 Et_2O/light petroleum, two successive developments) to afford pure **8c** (0.007 g, 12%) as a colourless solid.

Procedure B: To a stirred solution of **5** (0.046 g, 0.20 mmol) in dry DMF (0.6 mL) in an argon atmosphere was added a solution of NBS (0.035 g, 0.20 mmol) in dry DMF (0.6 mL). The resulting mixture was stirred at 20–25 °C for 149 h and then temperature was raised to 30 °C. After 10 days the temperature was raised at 35 °C and stirring was continued for 77 h. A new portion of NBS (0.018 g, 0.10 mmol) in dry DMF (0.3 mL) was added. After 15 days temperature was raised at 45 °C and stirring continued for 2 days. After stirring for two additional days at room temperature, mixture was diluted with EtOAc (15 mL). Organic solution was washed with 5% $NaHCO_3$ (20 mL), water and brine, dried and concentrated. Residue was purified by preparative TLC (3:2 Et_2O/light petroleum) to give

impure **8c** that was additionally purified by preparative TLC ($^{i}Pr_2O$, two successive developments). Pure compound **8c** (0.018 g, 29%) was obtained as a colourless solid.

Colourless prisms, mp 147–151 °C (Et_2O/hexane), $[\alpha]_D = -21.0$ (*c* 0.2, $CHCl_3$), $R_f = 0.60$ ($^{i}Pr_2O$). IR (film): ν_{max} 3449 (OH), 1783–1767 (C=O). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.86 (bs, 1H, OH), 4.31 (d, 1H, $J_{5,6} = 6.9$ Hz, H-5), 4.91 (t, 1H, $J = 6.5$ Hz, H-6), 5.59 (d, 1H, $J_{6,7} = 6.9$ Hz, H-7), 6.38 (d, 1H, $J_{2,3} = 5.6$ Hz, H-2), 7.29 (d, 1H, $J_{2,3} = 5.6$ Hz, H-3) 7.31–7.52 (m, 5H, Ph). NOE contact: H-3 and H-5. ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 53.52 (C-5), 78.21 (C-6), 83.11 (C-7), 110.03 (C-4), 122.18 (C-2), 126.64, 127.05, 128.78, 129.40, 134.04 (Ph), 148.83 (C-3), 168.66 (C-1). HRMS (ESI): *m/e* 328.0178 (M^++NH_4), calcd for $C_{13}H_{15}NBrO_4$: 328.0179.

4.1.12. General procedure for dehalogenation of iodo derivatives *3a, 4a, 4b, 7a and 8a*

Solution of 5-iodo derivatives **3a, 4a**, **4b**, **7a** and **8a** (1 equiv), AIBN (0.2 equiv) and Bu_3SnH (2 equiv) in dry toluene (0.05–0.06 mmol) was stirred in argon atmosphere at 100 °C for 1 h. The mixtures were evaporated and the residue purified by flash column chromatography (4:1 CH_2Cl_2/EtOAc; products **3**, **4** and **7**), or by preparative TLC (17:3 CH_2Cl_2/EtOAc; product **8**).

*4.1.12.1. (+)-crassalactone D (**3**).* Yield 81%. Colourless needles, mp 140–142 °C, (EtOAc/hexane), $[\alpha]_D = +20.5$ (*c* 0.2, EtOH),[1] $R_f = 0.63$ (5:2 EtOAc/hexane), Ref. [12] mp 138–140 °C, $[\alpha]_D = +13.6$ (*c* 0.2, EtOH). Both ^{1}H and ^{13}C NMR data of compound **3** are consistent with the naturally occurring (+)-crassalactone D [10] and its physical properties are in agreement with those reported in the literature [12].

*4.1.12.2. (4R)-crassalactone D (**4**).* Yields: 94% from **4a**, 95% from **4b**. Colourless needles, mp 137–142 °C, (EtOAc/hexane), $[\alpha]_D = +28.0$ (*c* 0.5, EtOH), $R_f = 0.61$ (5:2 EtOAc/hexane). ^{1}H and ^{13}C NMR data for **4** matched those previously reported by us [16].

*4.1.12.3. (7S)-(+)-crassalactone D (**7**).* Yield 93%. Colourless needles, mp 170–175 °C (CH_2Cl_2/hexane), $[\alpha]_D = +32.0$ (*c* 0.2, EtOH), $R_f = 0.24$ (Et_2O). ^{1}H and ^{13}C NMR data for **4** matched those previously reported by us [16].

*4.1.12.4. (4R,7S)-(+)-crassalactone D (**8**).* Yield 71%. Colourless needles, mp 139–141 °C (EtOAc/hexane), $[\alpha]_D = +73.0$ (*c* 0.2, EtOH), $R_f = 0.54$ (Et_2O). ^{1}H and ^{13}C NMR data for **4** matched those previously reported by us [16].

4.2. MTT assay

The colorimetric MTT assay was carried out following the reported procedure [32].

4.3. Cell cycle analysis

After treatment K562 cells were washed in cold PBS, fixed 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.

[1] Our optical rotation value is slightly greater than the data for $[\alpha]_D$ reported in ref 10 {$[\alpha]_D$=+7 (*c* 0.2, EtOH)}, but were in reasonable agreement with the reported data for synthetic (+)-crassalactone D reported in ref 12.

4.4. Detection of apoptosis

Apoptosis of K562 cells was evaluated with an Annexin V-FITC detection kit. Cells from each sample were collected (800 rpm/5 min, Megafuge 1.0R, Heraeus, Thermo Fisher Scientific) and pellet was re-suspended in 1 mL 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 1×10^6 cells/mL. The cell suspension (100 μL) was transferred to 5 mL 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. [33].

4.5. 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 investigated proteins was measured by densitometry and compared with control sample.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.12.011.

These data include additional characterization and biological data, as well as copies of ^{1}H, and ^{13}C NMR spectra of all final compounds. Crystallographic results are also included in this section. Supplementary data associated with this article can be found in online version.

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