Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 2629–2632

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

New Multidrug Resistance Modulators from Atractylodis Lanceae Rhizoma

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Received 7 August 2000; accepted 14 September 2000

Abstract—Three new oligoacylated sucroses designated atractysucroses-I, -II, and -III were obtained from Atractylodis Lanceae Rhizoma as multidrug resistance (MDR) modulating substances against human carcinoma cell lines and their chemical structures were characterized as a mixture of acyl groups. Atractysucrose-I modulates MDR in KB-C2 cells as strongly as verapamil. This is the first example of an MDR-modulator classified as a carbohydrate. © 2000 Elsevier Science Ltd. All rights reserved.

The development of multidrug resistance (MDR), which is observed as a responsiveness to chemotherapy, is one of the most serious problems in the treatment of cancer.[1] The major documented mechanism for tumor cells to acquire this MDR phenotype is the overexpression of an ATP-dependent membrane glycoprotein named P-glycoprotein (P-gp), which serves as an efflux pump for antitumor agents.[2,3] During the past decade, numerous compounds which restore the inherent potency of antitumor agents by inhibiting the action of P-gp have been found.[4] However, many of them found from in-house libraries have been accompanied with undesired side-effects due to their original efficacy. On this basis, we have engaged in a search for novel MDR modulators to inhibit the action of drug-effluxing membrane protein selectively from natural sources.[5] This paper communicates new MDR-modulating oligoacylated sucroses designated as atractysucroses-I (**1**), -II (**2**), and -III (**3**) from Atractylodis Lanceae Rhizoma (So-jutsu in Japanese) (Chart 1).

The MeOH extract of Atractylodis Lanceae Rhizoma showed growth inhibition against P-gp mediated MDR tumor cells (KB-C2)[6] in the presence of 0.1 μg/mL of colchicine. The MeOH extract was subjected to bioassay-guided separation (a growth inhibitory assay against KB-C2 cells in the presence of colchicine). After water–AcOEt partition, the active AcOEt layer was separated successively by normal and reversed-phase column chromatography and reversed-phase HPLC to furnish atractysucroses-I (**1**, 0.077%), -II (**2**, 0.017%), and -III (**3**, 0.026%) as MDR-modulating constituents. The IR spectrum of atractysucrose-I (**1**) exhibited absorption bands due to hydroxyl (3470 cm^{-1}) and ester carbonyl (1746 cm^{-1}) functions. The FAB-MS of **1** provided a quasimolecular ion peak at m/z 785 $(M+Na)^+$, the high-resolution MS measurement of which established the molecular formula of $C_{37}H_{62}O_{16}$. Although detailed analysis of the NMR data of **1** revealed that **1** was a mixture of three congeners with different distribution of acyl residues, appropriate conditions for separation could not be found. The chemical structure of atractysucrose-I (**1**) was, therefore, elucidated as a mixture. Intensive interpretation of the ^1H and ^{13}C NMR data of **1** (Tables 1 and 2)[7] in accordance with the HMQC experiment demonstrated the presence of seven oxymethine, three oxymethylene, one acetal tertiary and one acetal quaternary carbons. The H–H COSY spectrum of **1** allowed construction of two partial structures, one of which corresponds to the C-1 to C-6 fragment and the other of which is a portion from C-3′ to C-6′. The two partial structures could be connected by the following long-range C–H correlation: 1-H to C-5 and C-2′, 1′-H_2 to C-2′, 5′-H to C-2′, and 3′-H to C-2′ in the HMBC spectrum of **1**. Furthermore, all protons and carbons were definitely assigned with the aid of the H–H COSY, HMQC, and HMBC spectra. As a result, it was clarified that atractysucrose-I (**1**) is constructed from sucrose and two kinds of acyl groups, isovaleryl (major) and 2-methylbutyryl (minor).

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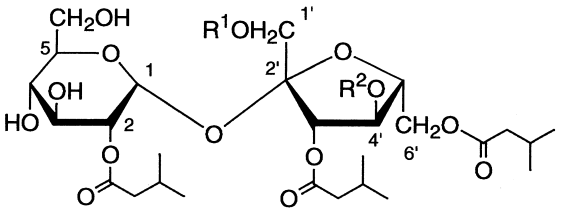
PII: S0960-894X(00)00536-9

Methanolic KOH treatment of **1** provided a sugar and a mixture of isovaleric and 2-methylbutyric acids. The sugar component was identified as sucrose by comparison of the optical rotation and NMR data with those of an authentic sample. The absolute configuration in 2-methylbutyric acid was determined to be *S* by GC analysis using β-cyclodextrin derived capillary column (β-DEX 325, Supelco Co. Ltd). The $^{3}J_{C-H}$ correlation between the acyl carbonyl carbons (δ_C 171.9–172.1 and 173.0–173.1) assignable to the isovaleryl residue and the 2-H, 1′-H, 3′-H, 4′-H, and 6′-H protons in **1** established the position of the isovalerylated hydroxyl groups in **1**. On the other hand, the two carbonyl signals at δ_C 175.6 and 175.8, which were correlated with the α-methine protons (δ 2.43 and 2.39) of the 2-methylbutyryl functions, were also correlated with the 1′-H oxymethylene (δ 4.01 and 4.09) and 4′-H oxymethine (δ 5.47) protons, respectively. Further analysis of the ^{1}H NMR spectrum of **1** clarified it to be a mixture of **1a**, **1b**, and **1c** in a ratio of 4:3:3.[8]

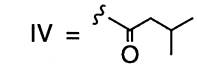


atractysucrose-I (**1**)

	R^1	R^2
1a	IV	IV
1b	2MB	IV
1c	IV	2MB

1a : 1b : 1c
(4 : 3 : 3)

IV =



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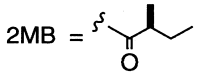

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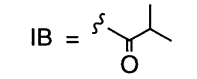

atractysucrose-II (**2**)

	R^1	R^2
2a	IB	IV
2b	IB	2MB
2c	IV	IB
2d	2MB	IB

2a + 2c : 2b + 2d
(6 : 4)

atractysucrose-III (**3**)

	R^1	R^2
3a	IV	H
3b	2MB	H

3a : 3b
(6 : 4)

Chart 1.

The physicochemical features of atractysucrose-II (**2**) in the NMR and IR spectra were significantly similar to those of **1**, whereas the FAB-MS of **2** afforded a

Table 1. NMR data for sugar moiety of atractysucroses-I (**1**) and -III (**3**)[a]

	^{1}H NMR		^{13}C NMR	
	1	**3**	**1**	**3**
Glucose moiety				
1	5.59 (d, 3.7)	5.47 (d, 3.7)	89.9	89.8
2	4.72 (dd, 3.7, 10.4)	4.73 (dd, 3.7, 10.4)	72.0	72.3
3	3.88 (dd, 9.2, 10.4)	3.86 (dd-like, ca. 10,10)	71.5	71.9
4	3.56 (dd, 9.2, 9.8)	3.52 (dd, 9.2, 9.8)	70.6	71.5
5	3.97 (ddd, 2.4, 4.9, 9.8)	3.92 (m)	72.5	72.4
6a	3.95 (dd, 2.4, 11.6)	3.89 (m)	62.6	62.6
6b	3.83 (dd, 4.9, 11.6)	3.80 (m)		
Fructose moiety				
1′a	4.07 (d, 12.2)	4.03 (d, 12.2)	63.0	63.2
1′b	4.02 (d, 12.2)	3.99 (d, 12.2)		
2′			102.7	102.8
3′	5.54 (d, 7.9)	5.23 (d, 7.9)	74.7	78.4
4′	5.47 (dd, 7.3, 7.9)	4.30 (dd, 7.9, 7.9)	73.7	74.0
5′	4.17 (ddd, 3.7, 7.3, 7.3)	4.13 (ddd, 3.7, 7.9, 7.9)	78.2	80.2
6′a	4.45 (dd, 7.3, 11.6)	4.53 (dd, 7.9, 12.2)	63.9	64.9
6′b	4.24 (dd, 3.7, 11.6)	4.24 (dd, 3.7, 12.2)		

[a] ^{1}H- and ^{13}C NMR spectra were taken in $CDCl_3$ at 500 and 125 MHz, respectively.

Table 2. NMR data for acyl moiety of atractysucroses-I (**1**) and-III (**3**)[a]

	^{1}H NMR		^{13}C NMR	
	1	**3**	**1**	**3**
Isovaleryl moiety				
CO			173.0–173.1	173.3–173.8
			171.9–172.1	172.2
α	2.15–2.30 (m)	2.28–2.32 (m)	42.6–43.0	43.0–43.1
		2.22–2.26 (m)		
β	2.00–2.15 (m)	2.11 (m)	25.5–25.6	25.6–25.8
γ	0.91–0.98 (m)	0.93–1.00 (m)	22.2–22.3	22.3–22.7
2-Methylbutyryl moiety (R^1)				
α	2.44 (ddq-like, ca. 7, 7, 7)	2.43 (ddq-like, ca. 7, 7, 7)	175.6	175.7
β	1.69 (ddq-like, ca. 7, 14, 7)	1.70 (ddq-like, ca. 7, 14, 7)	40.8	40.9
	1.49 (ddq-like, ca. 7, 14, 7)	1.50 (ddq-like, ca. 7, 14, 7)	26.6	26.7
α-Methyl	1.17 (d, 7.3)	1.17 (d, 6.7)	16.3	16.5
γ	0.91−0.98 (m)	0.92 (t, 7.3)	11.5	11.5
2-Methylbutyryl moiety (R^2)				
α	2.39 (ddq-like, ca. 7, 7, 7)		175.8	
β	1.68 (ddq-like, ca. 7, 14, 7)		40.7	
	1.48 (ddq-like, ca. 7, 14, 7)		26.5	
α-Methyl	1.13 (d, 7.3)		16.3	
γ	0.88 (t, 7.3)		11.4	

[a] ^{1}H and ^{13}C NMR spectra were taken in $CDCl_3$ at 500 and 125 MHz, respectively.

quasimolecular ion peak at m/z 771 $(M+Na)^+$ indicative of loss of one methylene unit relative to **1**. This presumption was also supported by HR-FAB-MS measurement, which established the molecular formula of $C_{36}H_{60}O_{16}$. Thus, atractysucrose-II (**2**) was deduced to be an isomer of **1** with different acyl composition. The NMR data of **2** closely resembled those of **1** except for the signals due to isovaleryl groups. Detailed analysis of the NMR properties of **2**, including the H–H COSY and HMQC spectra, indicated the presence of not only four congeners but also one molar isobutyryl group instead of an isovaleryl group.[9] The long-range C–H correlations between the carbonyl carbons in the acyl residues and the oxymethine and oxymethylene protons [isovaleryl: δ_C 171.9–172.1, 173.0 and δ 2.20–2.31 (α-H), 4.00, 4.10 (1′-H), 5.54, 5.55, 5.56, 5.57 (3′-H), 5.50 (4′-H), 4.23, 4.24. 4.47, 4.48 (6′-H), 4.73 (2-H); 2-methylbutyryl: δ_C 175.6, 175.8 and δ 2.44, 2.39 (α-H), 4.01, 4.09 (1′-H), 5.47 (4′-H); isobutyryl: δ_C 176.0, 176.2 and δ 2.62, 2.57 (α-H), 4.03, 4.09 (1′-H), 5.49 (4′-H)] in the HMBC spectrum of **2** defined the distribution of three kinds of acyl functions. In combination with chiral GC analysis of 2*S*-methylbutyric acid, atractysucrose-II (**2**) was elucidated to be a mixture of **2a** + **2c**:**2b** + **2d** in a ratio of 6:4.[8]

The quasimolecular ion peak at m/z 701 $(M+Na)^+$ and the molecular formula of $C_{32}H_{54}O_{15}$ for atractysucrose-III (**3**) obtained by respective FAB-MS and HR-FAB-MS measurement indicated lack of one isovaleryl function as compared with **1**. The NMR data of **3** showed that it was composed of two isomers distinguished by acyl residues. In addition, the proton signal ascribable to 4′-H of **3** was shifted to upper field, while the two carbon signals due to C-3′ and C-5′ of **3** submitted to downfield shift, as compared with those of **1**. These findings enabled us to elucidate **3** to be a 4′-deacylcongener of **1**.[10,11] Determination of the absolute configuration in 2-methylbutyric acid by chiral GC analysis as well as the location of the acyl residues [isovaleryl: δ_C 173.3–173.8 and δ 2.22–2.26, 2.28–2.32 (α-H), 3.98, 4.04 (1′-H), 5.23 (3′-H), 4.23, 4.24, 4.52, 4.53 (6′-H), 4.73 (2-H); 2-methylbutyryl: δ_C 175.7 and δ 2.43 (α-H), 3.99, 4.04 (1′-H)] by the HMBC experiment gave rise to the chemical structure of **3** as a mixture of **3a**:**3b** in a ratio of 6:4.[8]

Table 3. Reversal of MDR in KB-C2 cells by atractysucroses-I (**1**), -II (**2**) and -III (**3**)[a]

No.	Dose (μg/mL)	Growth inhibition (%)	
		KB-3-1[b]	KB-C2[c]
1	10	95±2	96±1
	3	14±8	86±3
	1	11±8	57±7
2	10	94±2	96±1
	3	13±12	83±4
	1	6±15	38±12
3	10	20±6	33±8
	3	19±10	32±10
	1	17±9	23±15

[a]Each value presents mean±s.d. Colchicine shows no cytotoxicity against KB-C2 cells at 0.1 μg/mL dose.
[b]Cytotoxicity of each compound.
[c]Growth inhibition in the presence of colchicine (0.1 μg/mL).

Assessment of MDR-modulating activity was carried out in the following manner. The extracts, fractions, and compounds were tested for their ability to restore cytotoxicity of colchicine against KB-C2 cells. After the cells were exposed to the tested samples in the presence of 0.1 μg/mL of colchicine, cell survival was assayed by MTT conversion. The biological outcome of atractysucroses-I (**1**), -II (**2**), and -III (**3**) is listed in Table 3. From those results, it appears that **1** and **2** with five acyl residues show more potent MDR-modulating activity than **3** with four acyl residues, and similar activities of **1** and **2** indicate that such slight difference in acyl composition would not affect the biological activity.

Finally, the modulating efficacy of atractysucrose-I (**1**), obtained as the major constituent among the three relatives, was compared with verapamil,[12] a standard MDR-modulator. The ability to restore sensitivity to colchicine was evaluated as an increase of the intracellular concentration of colchicine. As shown in Fig. 1, atractysucrose-I (**1**) modulates MDR in KB-C2 cells as strongly as verapamil.[13]

In summary, we have characterized new MDR-modulators, designated as atractysucroses-I (**1**), -II (**2**), and -III (**3**) from So-jutsu under bioassay-guided separation. To our knowledge, this is the first example of a P-gp mediated MDR-modulator, which was classified into carbohydrate. P-gp acts as an efflux pump of intracellular drugs. Considering that So-jutsu was prescribed in a number of Chinese medicinal preparations

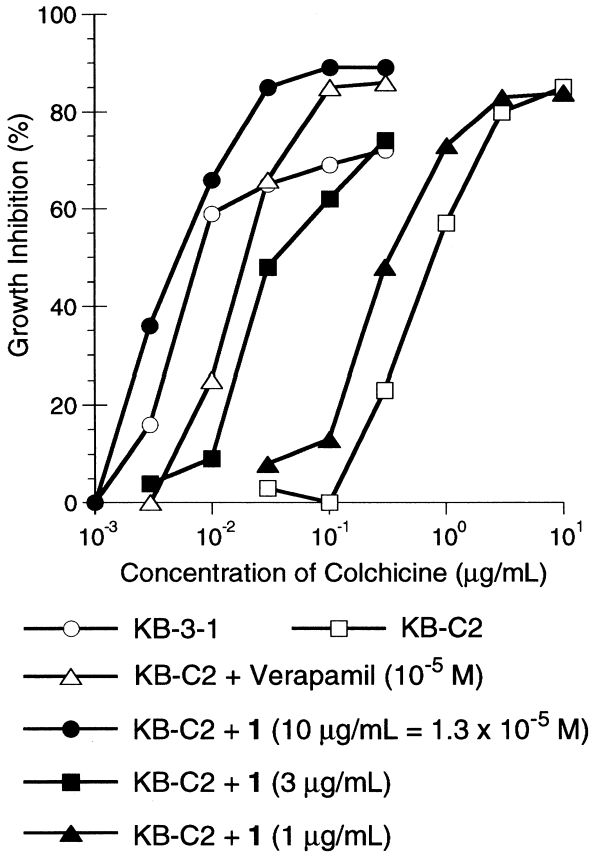

Figure 1. Reversal of MDR in KB-C2 cells by atractysucrose-I (**1**).

(Kampo-hozai), it should be noted that atractysucroses-I (**1**), -II (**2**), and -III (**3**), contained in the crude drug in comparatively high yield, might act as an enhancer of intracellular concentrations of other active substances. The structure–activity relationship in MDR-modulating activity of these oligoacylated disaccharides is currently under investigation in our laboratory.

Acknowledgements

The authors are grateful to the Naito Foundation, the Houansha Foundation, and the Ministry of Education, Science, Sports, and Culture of Japan for financial support.

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7. Besides the ^{1}H NMR data listed in Table 1, some following distinguishable signals appeared. **1**: δ 4.09 (d, J=12.2 Hz), 4.08 (d, J=12.2, 1′a-H), 4.01 (d, J=12.2, 1′b-H), 5.55 (d, J=7.9, 3′-H), 4.45 (dd, J=7.3, 11.6, 6′a-H), 4.23 (dd, J=3.7, 11.6, 6′b-H). **3**: δ 4.04 (d, J=12.2 Hz, 1′a-H), 3.98 (d, J=12.2, 1′b-H), 4.52 (dd, J=7.9, 12.2, 6′a-H), 4.23 (dd, J=3.7, 12.2, 6′b-H).
8. The ratio of each congener in atractysucroses-I (**1**), -II (**2**), and -III (**3**) was clarified by integration for methine and methylene protons linked to the acyl-carbonyl groups in the ^{1}H NMR spectra.
9. The NMR data for atractysucrose-II (**2**) was superimposable with that of **1** except for the signals assignable to the isobutyryl moiety. The signals ascribable to the isobutyryl moiety are as follows. ^{1}H NMR: δ 2.57, 2.62 (total 1H, m, α-H), 1.11–1.23 (m, β-H); ^{13}C NMR: δ_C 176.2, 176.0 (total 1C, C=O), 33.9, 33.7 (total 1C, α-C), 18.9, 18.8, 18.7 (total 2C, β-C).
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13. As shown in Fig. 1, the incubation with 10 μg/mL of **1** in the presence of low concentration of colchicine (1×10^{-3} μg/mL) exhibited no cytotoxicity against KB-C2. However, the 10 μg/mL concentration of **1** was cytotoxic against parental KB-3-1 cells (Table 3). These findings might be ascribable to specific cytotoxic potency of **1** against KB-3-1 cells.