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Synthesis of DAAS Derivatives and Their Enhancement of HL-60 Leukemia Cell Differentiation

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DAAS is the diacetoxy acetal derivative of a-santonin and induces HL-60 cell differentiation into granulocytes. In this report, we investigated the structure-activity relationship (SAR) of DAAS derivatives in the differentiation of human HL-60 leukemia cells. Although its derivatives themselves had less effect on HL-60 cell differentiation than DAAS, the monoacetyl derivative, **2**, mainly induced HL-60 cell differentiation. Moreover, compound **2** synergistically enhanced all-*trans* retinoic acid (ATRA)-induced HL-60 cell differentiation when combined with 50 nM ATRA, a well-known differentiation inducer. This enhancing effect is similar to that of DAAS in ATRA-induced differentiation.

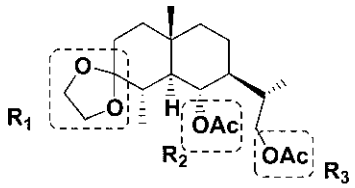
Key words: DAAS, Santonin, Leukemia cell differentiation, ATRA

INTRODUCTION

Leukemia is a cancer that originates from the bone marrow and develops when leukocytes undergo transformation into malignant cells. Terminal differentiation of leukemia cells can be induced by a variety of chemical and biological agents, indicating that the malignant state is not irreversible. Certain cancers may eventually be treated with agents that induce terminal differentiation, presumably with less morbidity than that produced by cytodestructive agents (Beere *et al.*, 1993).

Recently, several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic activity (Hehner *et al.*, 1999; Hall *et al.*, 1988). Some natural sesquiterpenes including vernolepin (Kupchan *et al*, 1968) and costunolide (Ohnishi *et al.*, 1997) were reported to have remarkable anti-leukemic activity *in vitro* and to have an α-methylene-γ-lactone moiety, which is expected to function as a major structural feature. There has been an increasingly large amount of research devoted to the development of new antitumor agents in this field.

More recently, we found that DAAS (Fig. 1), the noble acetal analogue of α-santonin that contains a non-lactone ring, has remarkable anti-leukemic activity, inducing HL-60 cell differentiation via down-regulation of NF-κB binding activity (Kim *et al.*, 2006). It also exhibits synergistic action with vitamin D_3 (Kim *et al.*, 2006).



DAAS

Fig. 1. Structure & derivatives of DAAS

Based on these interesting cell differentiation effects of DAAS, we synthesized some derivatives of DAAS and investigated the SAR of the DAAS derivatives in cellular differentiation in the human myelocytic leukemia HL-60 cell culture system. In addition, we examined the effects of combining DAAS or its active monoacetoxy acetal derivative with ATRA on HL-60 cell differentiation.

MATERIALS AND METHODS

Chemistry

Melting points were determined on a Mel-temp II laboratory device and are uncorrected. Nuclear magnetic reson-

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ance (NMR) data for ^{1}H-NMR were collected on the Varian UNITY *plus* 300 spectrometer and are reported in δ ppm downfield from tetramethylsilane (TMS). The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet. IR spectra (IR) were determined neat or from KBr disks on the Jasco FT-IR instrument and are reported in reciprocal centimeters. Thin layer chromatography (TLC) was carried out using plates precoated with silica gel 60F 254 that were purchased from Merck. Test compounds were synthesized by known methods and identified by m.p., IR spectra and ^{1}H-NMR data.

Synthesis of known compounds DAAS, 1, 2, 3 and 4

As shown in Scheme 1, the known DAAS derivatives were readily synthesized from tetrahydrosantonin (TS) using the previously reported procedures. Acetalization of TS using ethylene glycol and *p*-toluenesulfonic acid and its sequential reduction by lithium aluminum hydride gave diol compound (**1**), mp 150-152ºC (Kato *et al*., 1971; mp 149-151ºC), which was acetylated using Ac_2O and pyridine at rt to give the 13-α-monoacetate (**2**) and at 100ºC to give diacetate (DAAS), mp 84-86ºC (Kato *et al*., 1971; mp 83-85ºC). DAAS was partially deacetylated using 5%-KOH solution to give 6-α-monoacetate (**3**), mp 155-158ºC (Kato *et al*., 1971; mp 150-152ºC). DAAS was also deacetalized using AcOH to give oily compound (**4**) (Shibata *et al*., 1986).

(11S)-3,3-(Propylenedioxy)eudesmano-13,6α-lactone (5a)

A mixture of TS (7 g, 27.96 mmol), 1,3-propandiol (50 mL, 670.96 mmol) and *p*-toluenesulfonic acid (0.3 g, 1.57 mmol) in dry benzene was refluxed in a flask equipped with a Dean-Stark column for 12 h. Subsequently, the reaction mixture was cooled and washed with sodium bicarbonate solution. The benzene layer was separated and washed with saturated NaCl solution and dried over $MgSO_4$. The residue was purified by column chromatography (Hexane:EtOAc = 20:1) to give the lactone, **5a** (4.5 g, 54.67%); mp 170-172ºC; IR (KBr) 1773 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.97 (3H, s, CH_3), 1.22 (3H, d, *J* = 6.9 Hz, CH_3), 1.25 (3H, d, *J* = 6.6 Hz, CH_3), 3.78 (1H, m, CHO), 3.98 (4H, m, O$\underline{CH_2}CH_2\underline{CH_2}$O); $[\alpha]_D^{20}$: +13.60º (c= 1.00, MeOH).

(11*S*)-3,3-(Propylenedioxy)eudesmano-6,13-diol (5b)

The solution of lactone **1a** (1.88 g, 5.59 mmol) in dry THF 5 mL was carefully added to a mixture of lithium aluminum hydride (0.46 g, 12.19 mmol) in dry THF. The reaction mixture was stirred and refluxed for 1 h and then decomposed with ethyl acetate and H_2O. The solvent was removed and the residue was purified by silica gel column chromatography (Hexane:EtOAc = 2:1) to give **5b** as an diol (0.91 g, 52.12%) as white solid, mp 145-146ºC; IR (KBr) 3343 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.90 (3H, d, *J* = 7.2Hz, >CHCH_3), 0.92 (3H, s, CCH_3), 1.35 (3H, d, *J* = 6.9 Hz, CH_3), 3.55 (3H, m, CH_2OH, CHOH), 3.87 (4H, t, *J* = 5.7, O$\underline{CH_2}CH_2\underline{CH_2}$O); $[\alpha]_D^{20}$: -29.26º (c =1.00, MeOH).

(11*S*)-3,3-(Propylenedioxy)eudesmano-6-ol-13-acetate (5)

A solution of the diol, **5b** (0.07 g, 0.224 mmol), in acetic anhydride (1 mL) and pyridine (2 mL) was stirred at rt for 1 h and extracted with large amounts of ether. The ethereal solution was washed with sat. $NaHCO_3$, water, 2N-HCl, NaCl solution and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (Hexane:EtOAc = 6:1) to give **5** as an oil (0.08 g, 100%): IR (KBr) cm^{-1} 3395, 1713; ^{1}H-NMR (300 MHz, $CDCl_3$); δ 0.86 (3H, s, -C-CH_3), 0.87 (3H, d, *J* = 7.2 Hz, >CH-CH_3), 1.26 (3H, d, *J* = 6.6 Hz, >CH-CH_3), 2.04 (3H, s, $COCH_3$), 3.45 (1H, m, 6-H) 3.52 (2H, m, CH_2OAc), 3.78 (4H, m, O$\underline{CH_2}CH_2\underline{CH_2}$O); $[\alpha]_D^{20}$: +0.66º (c =1.00, MeOH).

(11*S*)-3,3-(Propylenedioxy)eudesmano-6,13-diacetate (6)

A solution of the diol, **5b** (0.14 g, 0.39 mmol), in acetic anhydride (4 mL, 9mmol) and pyridine (4 mL) was heated at 100ºC overnight and extracted with large amounts of ether. The ethereal solution was washed with sat. $NaHCO_3$, water, 2N-HCl, NaCl solution and dried over $MgSO_4$. After removal of the solvent, the residue was purified by silica gel column chromatography (Hexane:EtOAc = 6:1) to give **6** as an oil (0.04 g, 25.64%): IR(KBr) cm^{-1} 1731; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.87 (3H, d, *J* = 6.9 Hz, >CH-CH_3), 0.90 (3H, s, -C-CH_3), 0.98 (3H, d, *J* = 6.6 Hz, >CH-CH_3), 2.04 (3H, s, $COCH_3$), 2.02 (3H, s, $COCH_3$), 3.79 (2H, m, CH_2OC), 3.99 (4H, m, O$\underline{CH_2}CH_2\underline{CH_2}$O), 4.94 (1H, d, *J* = 10.05, CHOAc); $[\alpha]_D^{20}$: -13.46º (c =1.00, MeOH).

Biology

The HL-60 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.). The synthetic compounds were dissolved in dimethylsulfoxide to make 100 mg/mL stock solutions. The solutions were diluted at least 1000-fold in growth medium, such that the final concentration of dimethylsulfoxide had no effect on the differentiation and proliferation of HL-60 cells.

Determination of cell differentiation

HL-60 cell differentiation was assessed by the nitroblue

tetrazolium reduction assay as previously described (Collins *et al*., 1979). This assay is based on the ability of phagocytic cells to produce superoxide upon stimulation with PMA. For this assay, 2×10^5 cells were harvested by centrifugation and incubated with an equal volume of 1% NBT dissolved in PBS containing 200 ng/mL of freshly diluted PMA at 37°C for 30 min in the dark. Cytospin slides were prepared and then examined for blue-black nitroblue diformazan deposits, indicative of a PMA-stimulated respiratory burst. At least 200 cells were assessed for each experiment.

Statistical analysis

Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were used to determine the statistical significance of differences between values for various experimental and control groups. A *P* value of <0.05 was considered significant.

RESULTS AND DISCUSSION

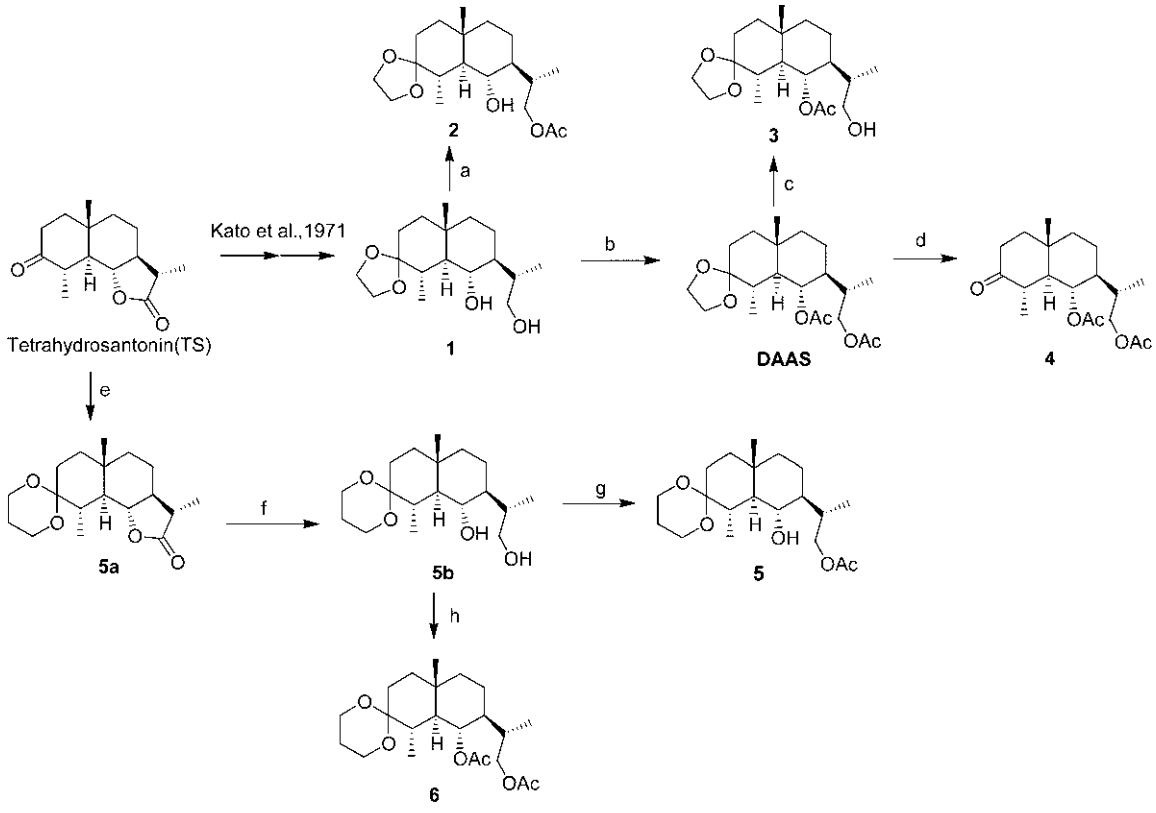
Chemistry

As shown in Scheme 1, the acetoxy acetal derivatives (**5** and **6**) were readily synthesized from α-santonin. The acetal **5a** was obtained from tetrahydrosantonin (TS) by acetalization using 1,3-propanediol and *p*-toluenesulfonic acid. The lactone **5a** was reduced using lithium aluminum hydride to give the diol **5b**, which was acetylated using Ac_2O or pyridine, depending on temperature, to give the monoacetate **5** and the diacetate **6**, respectively.

Effect of DAAS derivatives on HL-60 cell differentiation

HL-60 cells were seeded at a density of 2×10^5 cells/mL, and the cells were treated with medium alone or with 100 μg/mL of each of the DAAS derivatives for 72 h. Treatment with 100 ìg/mL DAAS induced HL-60 cell differentiation by approximately 53.7%, whereas treatment with tetrahydrosantonin did not. In this report, we further examined which functional group induced HL-60 cell differentiation. As shown in Table I, DAAS derivatives have different effectiveness in HL-60 cell differentiation. Among the derivatives, compounds **2** and **6** are less effective than DAAS in cell differentiation, while other derivatives have little or no effect on cell differentiation.

In order to determine whether or not the DAAS derivatives enhance HL-60 cell differentiation when combined with



Scheme 1. Synthetic pathway of DAAS derivatives

Table I. Effects of DAAS derivatives on HL-60 cell differentiation. HL-60 cells were treated with medium alone (M), with tetrahydrosantonin (TS), with DAAS or with 100 μg/mL of DAAS derivatives (**1**-**6**) for 72 h. Cell differentiation was assessed by the NBT reduction assay. Each value represents the mean±s.e. mean (n=3)

Compound (100 μg/mL)	% Differentiated cells
	Mean ± S.E. mean
M	1.6 ± 0.9
TS	1.2 ± 1.4
DAAS	*54.3 ± 3.9
1	2.6 ± 1.5
2	*36.2 ± 6.3
3	11.6 ± 1.7
4	6.5 ± 3.1
5	3.4 ± 2.9
6	* 25.3 ± 5.1

*P<0.001, relative to an untreated group (M).

low doses of ATRA, HL-60 leukemia cells were treated with 100 μg/mL of the compounds in combination with 50 nM ATRA. Cellular differentiation was then assessed by nitroblue tetrazolium reduction assays. As shown in Fig. 2A, the addition of compound **2** to cultures exposed to a suboptimal concentration of ATRA (50 nM), which by itself caused a relatively low level of differentiation, resulted in a marked increase in the degree of cell differentiation. Moreover, the enhancing effect of compound **2** is similar to that of DAAS in ATRA-induced differentiation. As shown in Fig. 2B and 2C, both DAAS and compound **2** by themselves induced HL-60 cell differentiation. In addition, both potentiated ATRA-induced HL-60 cell differentiation in a dose-dependent manner.

The SAR of DAAS derivatives on HL-60 cell differentiation

Among DAAS derivatives, DAAS was the most active in HL-60 cell differentiation. We earlier reported that chemical modification of its R_1 moiety to dithiacetal by replacing the oxygen atom with a sulfur atom as a bioisostere led to total loss of activity (Kim *et al.*, 2006). Now the newly synthesized propylenedioxy acetal **6**, which has an increased acetal ring size compared to DAAS, was found to be less active. Compound **4**, which is devoid of ethylenedioxy acetal from DAAS, also showed loss of activity. These results

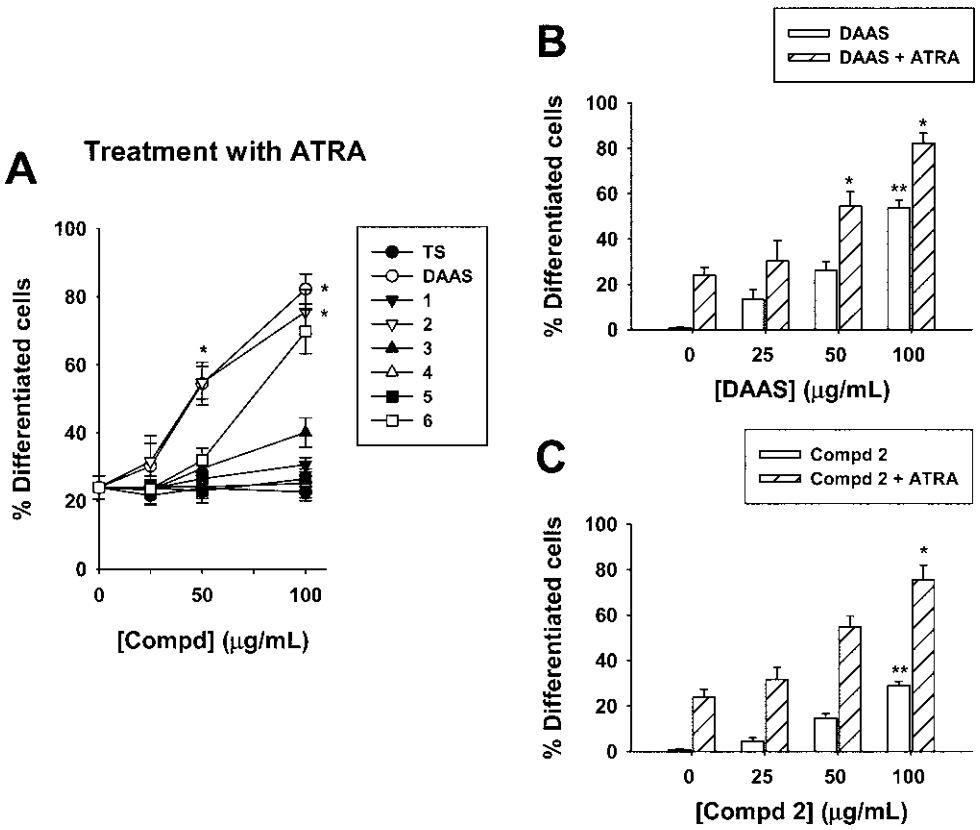


Fig. 2. Effects of DAAS derivatives on ATRA-induced HL-60 cell differentiation. HL-60 cells were treated for 72 h with 50 nM ATRA in combination with various concentrations (0-100 μg/mL) of tetrahydrosantonin (TS) or DAAS or with 100 μg/mL of DAAS derivatives (A). HL-60 cells were treated for 72 h with 50 nM ATRA alone or in combination with various concentrations (0-100 μg/mL) of DAAS (B) or compound 2 (C). Cell differentiation was assessed by the NBT reduction assay. Each value represents the mean±S.E. mean (n=3). *P<0.01 relative to the ATRA-treated group. **P<0.01 relative to the untreated group.

suggest that a moderately bulky ethylenedioxy acetal group on the R_1 moiety is optimal for activity. Compound **2**, deacetylated of the R_2 moiety of DAAS, had half the potency of DAAS. Also the monoacetyl acetal **3**, in which the R_3 acetyl moiety of DAAS was removed, exhibited a remarkable decrease in activity, while the diol **6** was devoid of activity. We already reported that the homologous analogue, in which an ethyl group was substituted for a methyl group in the R_3 moiety, or the carbamate analogue by conversion to NH_2 as a bioisostere showed remarkably decreased activity. So we conclude that both acetyl groups of DAAS are required for optimal activity and, in particular, the acetyl functional group of R_3 is more important than the R_2 acetyl group and is expected to play an important role in its cell differentiating activity.

Many previous studies have shown that chemical combinations involving ATRA exert an additive or synergistic effect on HL-60 cell differentiation (Hershberger *et al*., 2001; Kang *et al*., 2001). Here, we report the combination of DAAS or its monoacetoxy derivative **2** with ATRA affects HL-60 cell differentiation. Both DAAS and compound **2** potentiate the induction of HL-60 cell differentiation when used in combination with ATRA.

The mechanism by which DAAS potentiates ATRA-induced HL-60 cell differentiation is not clear. However, the results presented here suggest that treatment of patients with combinations of DAAS and ATRA may produce a greater therapeutic response than ATRA alone, possibly with less toxicity.

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