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# Synthesis and Immunosuppressive Activity of Novel Succinylacetone Analogues

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This study describes the synthesis of novel enol esters (**3**) and triketones (**4**) as analogues of succinylacetone (SA) (Ed- this abbreviation is introduced here based on your use of it in the body of the paper) and the evaluation on the mouse allogeneic mixed lymphocyte reaction (MLR) and the murine model of antigen-induced paw edema formation for immunosuppressive activity. Enol esters (**3a-f**) were about 2-4 fold more potent than SA in *in vitro* activity.

**Key words:** Immunosuppressive activity, Succinylacetone, Alkylacyloxy enol esters, Mixed lymphocyte reaction (MLR), Antigen-induced, Paw edema formation.

## INTRODUCTION

Immunosuppressive drugs are widely used in possible organ transplantation and treatment of autoimmune diseases. Azathioprine (Elison, *et al.*, 1975) was first introduced to control graft rejection and is still used in combination with other immunosuppressive agents. The discovery of cyclosporin A (CsA) (Borel, *et al.*, 1982) allowed major improvements in transplantation. It induced a dramatic increase of organ allograft survival which made possible liver and cardiac transplants. Recently, FK-506 (Tanaka, *et al.*, 1987) was found to be 10-100 fold more potent than CsA. These two immunosuppressive compounds block T-cell activation *via* the inhibition of interleukin-2 expression (O'Keefe, *et al.*, 1994). However, all these agents, as well as an important proportion of failures, show a number of undesired side effects including nephrotoxicity, hypertension, neurological disorders, gingival overgrowth, and hirsutism (Ed- the use of including has already indicated that this list is only partial) (Shaw, *et al.*, 1996; Perico, *et al.*, 1997). Therefore, there is a need for new, less toxic compounds with new mechanisms of action, which could lead to a more specific immunosuppression.

Succinylacetone (SA) is a seven carbon organic ketoacid. It is a new immunosuppressive compound that was first noted in the urine of patients with hereditary tyrosinemia due to a deficiency of fumarylacetoacetate hydrolase (Lindblad, *et al.*, 1977). The immunosuppressive effects of SA were discovered in its ability to inhibit tumor allograft rejection (Tschudy, *et al.*, 1981), marked immunosuppression in acute graft-*vs.*-host disease (GVHD) (Hess, *et al.*, 1987; Fidler, *et al.*, 1993), and experimental autoimmune uveitis (Skolik, *et al.*, 1988). We report here a part of our program aimed at the discovery of new SA analogues that will be more efficacious immunosuppressive agents. This will be achieved by varying the functional group components *via* the bioisosteric approach (Silverman, *et al.*, 1992) and imposing conformational constraints. For this purpose the SA structure can be divided into three domains (Fig. 1): (A) the 1,3-diketones; (B) the linking arm, and (C) the carboxyl group. SA derivatives were patented (Nitecki, *et al.*, 1990) for the treatment of GVHD or autoimmune diseases by changing the linking arm and the carboxyl part. The carboxyl group was displaced with methyl, trifluoromethyl, esters, amides, phosphonic esters, and tetrazole (Ed-

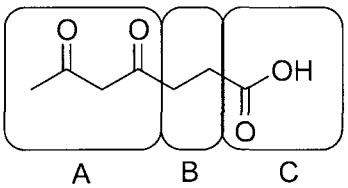


**Fig. 1.** Structure of succinylacetone

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confirm spelling) groups and the linking arm was varied with the elongation of the carbon chain. Among these SA derivatives, methyl ester ($MeCOCH_2COCH_2CH_2COOMe$) and polyethylene glycol amide ($MeCOCH_2COCH_2CH_2CO$-Pro-NH-PEG) of SA were more potent than SA in *in vitro* activity. With this consideration in mind, we mainly focused our investigation on methyl ester SA bearing, conformationally constrained, cyclopentanone and cyclohexanone in the section of 1,3-diketones of SA.

## MATERIALS AND METHODS

Unless otherwise noted, materials were obtained from commercial supplies and used without purification. All reactions requiring anhydrous conditions were performed in oven-dried glassware under $N_2$ atmosphere. Tetrahydrofurar (THF) was distilled from sodium-benzophenone immediately prior to use. Acetonitrile was distilled from phosphorus pentoxide (Ed- if the acetonitrile was also distilled just prior to use, then these two sentences could be better combined as, "Tetrahydrofuran (THF) and acetonitrile were distilled from sodium-benzophenone and phosphorus pentoxide, respectively, immediately prior to use."). Thin layer chromatography (TLC) was carried out using Merck Silica Gel 60 precoated plates. Products were purified by flash column chromatography on Merck 60 (230-400 mesh) silica gel. $^1H$ nuclear magnetic resonance (NMR) and $^{13}C$ NMR were recorded on Bruker AMX 300 and reported in ppm downfield from tetramethylsilane (δ 0.00) and $CDCl_3$ (δ 77.0), respectively.

### General procedure for the synthesis of enol esters 3

To a stirred solution of 1,3-cycloalkanedione (2 mmol) and triethylamine (2.2 mmol) in dry THF (3 mL), acyl chloride was added. The reaction mixture was stirred at room temperature for 1 h, then quenched with water, and extracted with ether. The organic layer was dried with magnesium sulfate and concentrated to give a practically pure enol ester, which was able to be used in the isomerization (Ed- this American spelling is much more common; although if desired the original is acceptable) reaction without further purification. Purification of crude product for the immunosuppressive activity test was performed by flash column chromatography.

**Methyl 3-oxo-1-cyclopenten-1-yl butanedioate (3a)**
Yield 55%; mp: 49-51; $R_f$ = 0.7 (ethyl acetate); $^1H$-NMR ($CDCl_3$) 6.22-6.23 (m, 1H), 3.72 (s, 3H), 2.83-2.89 (m, 2H), 2.75-2.79 (2H, m), 2.69-2.74 (m, 2H), 2.44-2.47 (m, 2H); $^{13}C$-NMR ($CDCl_3$) 206.5, 179.4, 172.2, 168.2, 116.6, 52.1, 33.3, 29.5, 28.7, 28.4; HRMS (EI) calculated for $C_{10}H_{12}C_5$ 212.0685, determined as 212.0651.

**Methyl 3-oxo-1-cyclohexen-1-yl butanedioate (3b)**
Yield 73%; $R_f$ = 0.8 (ethyl acetate); $^1H$-NMR ($CDCl_3$) 5.89-5.90 (m, 1H), 3.70 (s, 3H), 2.77-2.82 (m, 2H), 2.67-2.71 (m, 2H), 2.53-2.58 (m, 2H), 2.37-2.42 (m, 2H), 2.02-2.11 (m, 2H); $^{13}C$-NMR ($CDCl_3$) 199.5, 172.3, 169.6, 169.2, 117.8, 51.8, 36.4, 29.4, 28.5, 28.2, 21.2; HRMS (EI) calculated for $C_{11}H_{14}O_5$ 226.0841, determined as 226.0816.

**Methyl 3-oxo-1-cyclopenten-1-yl pentanedioate (3c)**
Yield 55%; mp: 37-39; $R_f$ = 0.7 (ethyl acetate); $^1H$-NMR ($CDCl_3$) 6.22-6.23 (m, 1H), 3.69 (s, 3H), 2.74-2.78 (m, 2H), 2.64 (t, 2H, $J$ = 7.3 Hz), 2.42-2.47 (m, 4H), 1.98-2.07 (m, 2H, $J$ = 7.32); $^{13}C$-NMR ($CDCl_3$) 205.8, 178.5, 172.0, 167.6, 115.4, 50.6, 32.5, 31.6, 30.6, 27.7, 18.5.

**Methyl 3-oxo-1-cyclohexen-1-yl pentanedioate (3d)**
Yield 58%; $R_f$ = 0.7 (ethyl acetate); $^1H$-NMR ($CDCl_3$) 5.90-5.91 (m, 1H), 3.69 (s, 3H), 2.52-2.59 (m, 4H), 2.38-2.45 (m, 4H), 1.95-2.11 (m, 4H); $^{13}C$-NMR ($CDCl_3$) 194.5, 168.1, 164.5, 112.5, 46.7, 31.7, 28.3, 27.6, 23.3, 16.2, 14.7; HRMS (EI) calculated for $C_{12}H_{16}O_5$ 240.0998, determined as 240.0992.

**3-Acetyloxy-2-cyclopenten-1-one (3e)**
Yield 40%; $R_f$ = 0.7 (ethyl acetate); $^1H$-NMR ($CDCl_3$) 6.22 (t, 1H), 2.76 (m, 2H) 2.45 (m, 2H), 2.29 (s, 3H); $^{13}C$-NMR ($CDCl_3$) 206.8, 179.5, 166.2, 116.2, 33.3, 28.7, 21.4; HRMS (EI) calculated for $C_7H_8O_3$ 140.0473, determined as 140.0458.

**3-Acetyloxy-2-cyclohexen-1-one (3f)**
Yield 65%; $R_f$ = 0.8 (ethyl acetate); $^1H$-NMR ($CDCl_3$) 5.90 (t, 1H), 2.54 (m, 2H), 2.40 (m, 2H), 2.23 (s, 3H), 2.07 (m, 2H); $^{13}C$-NMR ($CDCl_3$) 199.4, 169.6, 167.1, 117.2, 36.5, 28.0, 21.0, 21.0; HRMS (EI) calculated for $C_8H_{10}O_3$ 154.0630, determined as 154.0642.

**3-Oxo-1-cyclopenten-1-yl 4-methylbenzoate (3g)**
Yield 83%; $R_f$ = 0.5 (ethyl acetate/hexane 1:1); $^1H$-NMR ($CDCl_3$) 8.00 (m, 2H), 7.31 (m, 2H), 6.38 (t, 1H, $J$ = 1.5 Hz), 2.52 (m, 2H), 2.29 (m, 2H), 2.45 (s, 3H).

**3-Oxo-1-cyclohexen-1-yl 4-methylbenzoate (3h)**
Yield 61%; $R_f$ = 0.5 (ethyl acetate/hexane 1:1); $^1H$-NMR ($CDCl_3$) 7.93-7.96 (m, 2H), 7.25-7.27 (m, 2H), 6.07 (t, 1H, $J$ = 1.2 Hz), 2.66 (t, 2H, $J$ = 6.5 Hz), 2.41-2.45 (m, 2H), 2.41 (s, 3H), 2.08 (qu, 2H, $J$ = 6.5 Hz).

**3-Oxo-1-cyclopenten-1-yl 4-trifluoromethylbenzoate (3i)**
Yield 37%; $R_f$ = 0.5 (ethyl acetate/hexane 1:1); $^1H$-NMR ($CDCl_3$) 8.25 (d, 2H, $J$ = 8.2 Hz), 7.79 (d, 2H, $J$ = 8.2 Hz),

6.42 (t, 1H, $J$ = 1.6 Hz), 2.92-2.96 (m, 2H), 2.54-2.57 (m, 2H).

**3-Oxo-1-cyclohexen-1-yl 4-trifluoromethylbenzoate (3j)**

Yield 64%; $R_f$ = 0.5 (ethyl acetate/hexane 1:1); $^1$H-NMR ($CDCl_3$) 8.22 (d, 2H, $J$ = 8.0 Hz), 7.77 (d, 2H, $J$ = 8.5 Hz), 6.07 (t, 1H, $J$ = 1.2 Hz), 2.68-2.72 (m, 2H), 2.46-2.51 (m, 2H), 2.11-2.20 (m, 2H).

## General procedure for the synthesis of the triketones 4

To a stirred solution of the crude product, **3** (2 mmol), and potassium cyanide (2.2 mmol) in acetonitrile (3 mL), triethylamine (2.2 mmol) was added dropwise with a syringe. The reaction mixture was stirred at room temperature for 12 h, acidified with 1 N hydrochloric acid, and extracted with ether. After the ether layer was washed with aqueous sodium bicarbonate, the resulting aqueous layer was neutralized, extracted with ether, dried and concentrated to produce the final product.

**Methyl 4-(2,5-dioxocyclopentenyl)-4-oxobuanoate (4a)**

Yield 68%; $^1$H-NMR ($CDCl_3$) 13.9 (s, 1H), 3.70 (s, 3H), 3.24-3.29 (m, 2H), 2.76-2.78 (m, 2H), 2.62-2.73 (m, 2H), 2.52-2.55 (m, 2H).

**Methyl 4-(2,6-dioxocyclohexyl)-4-oxobuanoate (4b)** (Tang, *et al.,* 1998)

Yield 30%; $^1$H-NMR ($CDCl_3$) 17.65 (s, 1H), 3.70 (s, 3H), 3.39 (t, 2H, $J$ = 6.9 Hz), 2.62-2.70 (m, 4H), 2.49 (t, 2H, $J$ = 6.9 Hz), 1.95-2.04 (m, 2H); $^{13}$C-NMR ($CDCl_3$) 204.5, 196.9, 195.5, 173.3, 113.1, 51.8, 38.5, 36.5, 32.4, 27.8, 19.1.

**Methyl 4-(2,5-dioxocyclopentenyl)-4-oxopentanoate (4c)**

Yield 63%; $^1$H-NMR ($CDCl_3$) 13.75 (bs, 1H), 3.68 (s, 3H), 2.96-3.00 (m, 2H), 2.77-2.81 (m, 2H), 2.51-2.55 (m, 2H), 2.42-2.38 (m, 2H), 1.95-2.03 (m, 2H); $^{13}$C-NMR ($CDCl_3$) 203.6, 201.1, 199.9, 173.4, 114.4, 51.6, 37.9, 33.6, 33.1, 28.2, 18.8.

**Methyl 4-(2,5-dioxocyclopentanyl)-4-oxopentanoate (4d)**

Yield 50%; $^1$H-NMR ($CDCl_3$) 18.09 (s, 1H), 3.67 (s, 3H), 3.06-3.11 (m, 2H), 2.67-2.72 (m, 2H), 2.48-2.52 (m, 2H), 2.38-2.42 (m, 2H), 1.91-2.04 (m, 4H).

**2-Acetyl-1,3-cyclopentanedione (4e)**

Yield 20%; $^1$H-NMR ($CDCl_3$) 2.74 (bs, 2H), 2.53 (bs, 5H); $^{13}$C-NMR ($CDCl_3$) 204.1, 199.9, 198.7, 114.6, 33.7, 28.5, 25.8.

**2-Acetyl-1,3-cyclohexanedione (4f)**

Yield 32%; $^1$H-NMR ($CDCl_3$) 2.65-2.69 (m, 2H), 2.60 (s, 3H); 2.47-2.52 (m, 2H), 1.95-2.03 (m, 2H); $^{13}$C-NMR ($CDCl_3$) 203.2, 198.7, 195.5, 113.4, 38.6, 33.3, 28.8, 19.0.

**2-(4-Methylbenzoyl)-1,3-cyclopentanedione (4g)**

Yield 78%; mp: 102-103; $^1$H-NMR ($CDCl_3$) 7.98-8.02 (m, 2H), 7.25-7.29 (m, 2H), 2.56-2.60 (m, 2H), 2.80-2.84 (m, 2H), 2.42 (s, 3H).

**2-(4-Methylbenzoyl)-1,3-cyclohexanedione (4h)**

Yield 80%; mp: 69-70; $^1$H-NMR ($CDCl_3$) 16.90 (s, 1H), 7.44-7.46 (m, 2H), 7.18-7.21 (m, 2H), 2.70-2.74 (m, 2H), 2.48-2.52 (m, 2H), 2.39 (s, 3H), 2.03-2.10 (m, 2H).

**2-(4-Trifluoromethylbenzoyl)-1,3-cyclopentanedione (4i)**

Yield 90%; $^1$H-NMR ($CDCl_3$) 8.08-8.11 (m, 2H), 7.72-7.75 (m, 2H) 2.88-2.92 (m, 2H), 2.60-2.64 (m, 2H); $^{13}$C-NMR ($CDCl_3$) 206.9, 198.1, 192.1, 137.9, 130.5, 130.1, 125.4, 121.8, 113.6, 33.5, 28.6.

**2-(4-Trifluoromethylbenzoyl)-1,3-cyclohexanedione (4j)**

Yield 77%; $^1$H-NMR (acetone-$d_6$) 7.69-7.71 (m, 4H), 2.64 (bs, 4H), 2.04-2.12 (m, 2H).

**Mixed lymphocyte reaction (MLR) assay** (Meo, *et al.*, 1979)

Bidirectional MLR assay was adapted using spleen cells from C3H and C57BL/6 mice. Briefly, splenocytes were separated by Ficoll-Hypaque density gradient centrifugation. Each $1\times10^6$ cells were cocultured in 96 well plate in the absence or presence of serial dilutions of the tested compounds for 4 days. All compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted in the culture medium used for the assay. To take into account a possible interfering effect of DMSO, each experiment included a control in which the DMSO concentration was made equal to that in the compound treated cultures. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to determine the cell proliferation. $IC_{50}$ was determined by the concentration at which 50% of the cell proliferation were inhibited.

**Antigen-induced paw edema test** (Di Pierro, *et al.*, 1997)

Groups of BALB/c mice were immunized by two subcutaneous injections on days 0 and 7 of keyhole limpet hemocyanin (KLH, 100 μg/mouse) emulsified in 0.2 mL complete Freunds adjuvant (CFA). Nonimmunized mice (control) were treated with CFA alone. To evaluate the immunosuppressive activity, the compounds, **3b**, **3d**, SA, leflunomide (LEM), and CsA dissolved in corn oil, were

administrated daily from day 0 until day 13. On day 14 the immunized mice were challenged in the hind footpad with 20 μg of KLH suspended in physiological saline. Paw thickness were quantified eight hours after antigen challenge using a scientific micrometer. Scores of immune suppression were calculated by the following equation.

**Inhibition of paw edema formation (%) = 100 – (paw thickness of immunosuppressant treated group – normal paw thickness)/(paw thickness of antigen induced group – normal paw thickness)×100**

## RESULTS AND DISCUSSION

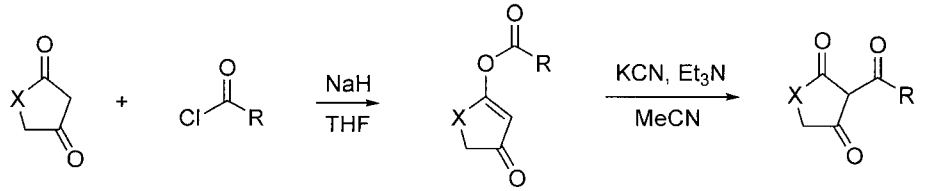
The synthesis of 2-alkanoyl or 2-benzoylcycloalkane-1,3-diketones (**4**), analogues of SA, is outlined in Scheme 1. Preparations of **4a-j** were accomplished by the cyanide catalyzed isomerization of enol esters (**3a-j**) derived from the reaction of the corresponding acyl chloride with 1,3-cyclopentanedione or 1,3-cyclohexanedione using triethylamine as a base in THF, as has been described in the literature (Akhrem, *et al.*, 1978; Montes, *et al.*, 1996). The effect of the enol esters (**3a-j**) and triketones (**4a-j**) on the mouse allogeneic MLR (Ed- already defined in the M&M section above) assay (Meo *et al.*, 1979; Kawai, *et al.*, 1993) was examined to investigate the relationship between the immunosuppressive activity and the structure. MLR assays are in vitro models of allogeneic T cell activation wherein peripheral blood mononuclear leukocytes (PBLs) from a single individual are exposed to a pool of mitomycin C-treated PBLs from randomly selected individuals. After 4 days, the incorporation of 3*H*-thymidine into cellular DNA is measured. Active immunosuppressants inhibit proliferation, thereby reducing the amount of thymidine incorporation when compared to untreated controls. The MLR assay is a fundamental benchmark test for immunosuppressants activity. Table I presents the $IC_{50}$ values for the compounds investigated in this study. The biological activity of these compounds was initially compared to that of SA ($IC_{50}$ = 1584 μ*M*). At initial examination, the $IC_{50}$ values of **4a** and **4b**, which possess cycloalkanone, were similar to that of SA in activity. As a result, this series was examined in more detail with one-more elongated analogues, **4c-d**, and methyl and substituted benzoyl analogues, **4e-j**. These compounds were also similar to SA in activity. Next, we examined the intermediate with enol esters (**3**). 3-Alkylacyloxy-2-cycloalken-1-one (**3a-f**) was 3 fold more potent than SA. Compound **3e**, with a methyl as R, was significantly more active than SA. Benzoyl substituents **3g-j** were less active than alkylacyloxy enol esters, indicating that the 1,3-diketone group is not necessary for the immunosuppressive activity.

The compounds **3b** and **3d**, more active *in vitro*, were tested *in vivo* (Table II) in a murine model of antigen-induced paw edema formation (Di Pierro, *et al.*, 1997). These compounds had more immunosuppressive activity than that of SA and LEM. LEM, an isoxazole derivative, is a new, disease-modifying, anti-rheumatic drug approved for the treatment of rheumatoid arthritis (RA), one of the autoimmune diseases (Sanders, *et al.*, 2002).

**Table I.** Mixed Leukocyte Response (MLR) data for test compounds

| Compound | X | R | MLR[a] |
|---|---|---|---|
| **3a** | $CH_2$ | $(CH_2)_2COOMe$ | 695 |
| **3b** | $(CH_2)_2$ | $(CH_2)_2COOMe$ | 460 |
| **3c** | $CH_2$ | $(CH_2)_3COOMe$ | 639 |
| **3d** | $(CH_2)_2$ | $(CH_2)_3COOMe$ | 604 |
| **3e** | $CH_2$ | Me | 887 |
| **3f** | $(CH_2)_2$ | Me | 398 |
| **3g** | $CH_2$ | 4-MePh | 2844 |
| **3h** | $(CH_2)_2$ | 4-MePh | 3704 |
| **3i** | $CH_2$ | 4-$CF_3$Ph | 2350 |
| **3j** | $(CH_2)_2$ | 4-$CF_3$Ph | 2830 |
| **4a** | $CH_2$ | $(CH_2)_2COOMe$ | 1876 |
| **4b** | $(CH_2)_2$ | $(CH_2)_2COOMe$ | 1367 |
| **4c** | $CH_2$ | $(CH_2)_3COOMe$ | 2019 |
| **4d** | $(CH_2)_2$ | $(CH_2)_3COOMe$ | 1060 |
| **4e** | $CH_2$ | Me | 1068 |
| **4f** | $(CH_2)_2$ | Me | 1435 |
| **4g** | $CH_2$ | 4-MePh | 1532 |
| **4h** | $(CH_2)_2$ | 4-MePh | 2293 |
| **4i** | $CH_2$ | 4-$CF_3$Ph | 2097 |
| **4j** | $(CH_2)_2$ | 4-$CF_3$Ph | 1247 |
| **SA** | – | – | 1584 |

[a] Mixed leukocyte response $IC_{50}$ (μM)




X = $CH_2$, $(CH_2)_2$ **3** **4**

**Scheme 1.** Synthetic approach to compounds **3** and **4**

**Table II.** Immunosuppressive Activity of **3b**, **3d**, Succinylacetone, Leflunomide, and Cyclosporin A on Antigen-Induced Paw Edema Formation in the Mouse

| Treatment | Scores[a] (mm) | Inhibition of paw edema formation (%) | Statistical significance (*p*-value) |
|---|---|---|---|
| Corn oil | 3.566 ± 0.355 | | |
| **3b** | 3.013 ± 0.395 | 47 | 0.0008 |
| **3d** | 3.050 ± 0.328 | 43 | 0.0003 |
| SA | 3.288 ± 0.295 | 23 | 0.0195 |
| LEM | 3.181 ± 0.288 | 32 | 0.0012 |
| CsA | 2.794 ± 0.210 | 65 | 0.005 > |

[a] Thickness of paw. Mean ± standard error.

In conclusion, our study on the analogues of SA demonstrated that the alkylacyloxy enol esters are a group of novel lead compounds as immunosuppressive agents. Compounds **3a-f** will be further studied in *in vivo* models of immunosuppression.

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