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SYNTHESIS OF NEW TNF-α INHIBITORS AND THEIR BIOLOGICAL PROPERTIES

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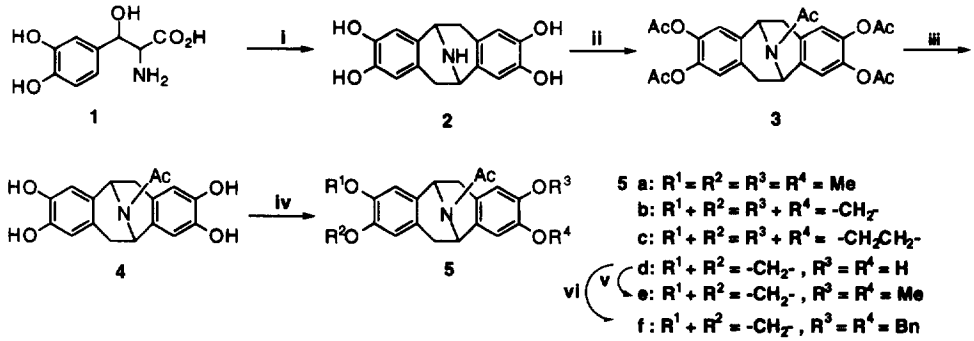
Abstract: New pavine alkaloid derivatives with various substitutions on their aromatic rings and nitrogen atom were prepared and evaluated for their inhibitory activity against TNF-α production in mouse macrophages stimulated with LPS. Some compounds showed potent inhibitory activities *in vitro* and protected mice against lethality of septic shock induced by LPS and D-galactosamine.

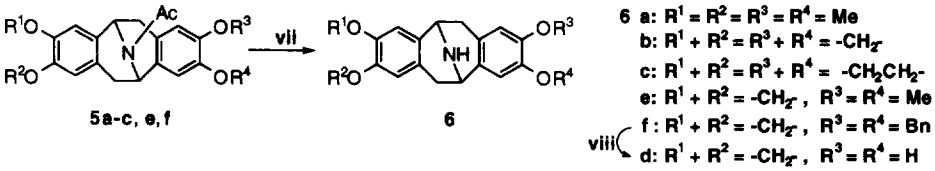
Introduction: Tumor necrosis factor-α (TNF-α) is a cytokine that mainly is produced by activated macrophages and that has a cytotoxic effect on some tumor cells and transformed cells. There is increasing evidence that TNF-α is a major mediator of inflammatory and immune responses and has pleiotropic biological effects.[1] TNF-α also has a crucial role in septic shock, and elevation of TNF-α level in circulatory system and/or tissue occurs in diseases such as cachexia, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), AIDS, and noninsulin-dependent diabetes mellitus(NIDDM).[1, 2] Moreover TNF-α is a strong inducer of such other pro-inflammatory cytokines as IL-1, IL-6, and IL-8.[2f] Agents which inhibit TNF-α production therefore could be effective for direct or indirect treatment of such diseases. The exact way TNF-α acts in the cytokine network of these diseases, however, is still not clear. We discovered that pavine-type[3] isoquinoline alkaloid derivatives are potent inhibitors of TNF-α production in LPS-stimulated mouse macrophages. These compounds given orally also inhibit LPS-induced septic shock in mice. The synthesis and structure activity-relationships of pavine alkaloid derivatives for *in vitro* and *in vivo* tests are described.

Chemistry: The compounds shown in Tables 1-3 were synthesized as outlined in Schemes 1-3. Compounds bearing various substitutions on their aromatic rings were prepared as shown in Scheme 1. Tetrahydroxypavinan (**2**),[4] the key intermediate of pavine structure was prepared directly from commercially available 3,4-dihydroxyphenylserine (**1**) by condensation in aqueous hydrochloric acid.[5] Peracetylation of **2** with excess acetic anhydride, followed by partial hydrolysis gave dicatechol derivative **4**. Alkylation or alkylenedioxylation[6] of **4** with various halide derivatives gave **5a-f**. Hydrolysis of acetamide **5a-c**, **5e**, and **5f** gave the respective secondary amino derivatives **6a**[7]**-c** ,**6e**, and **6f** (Scheme 2). Dibenzyl ether **6f** was converted to catechol **6d** by hydrogenolysis. Series of amino substituted compounds were prepared as shown in Scheme 3. Alkylation of **6b** with the requisite alkyl halides gave **7a**[5b, 8]**-d**, **7j**, **7r**, and **7t**. Deprotection of phthalimide **7r** and **7t** gave the respective primary amino derivatives **7s** and **7u**. Acylation of **6b** with the requisite acid chlorides gave **7f-i**, **7k**, **7m**, and **7o**. Reduction of the remaining ester functions of **7m** and **7o**

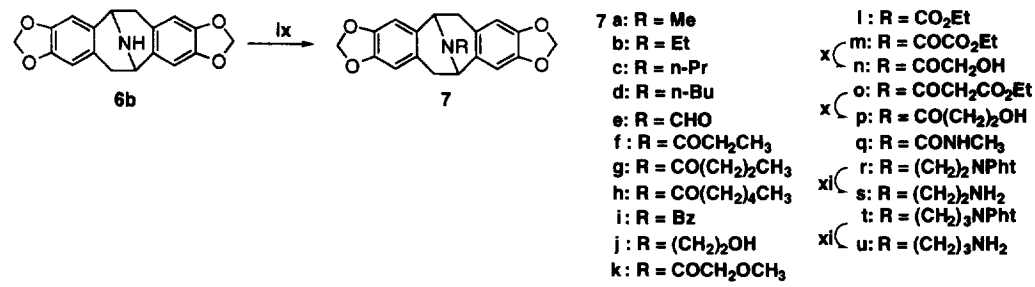
Scheme 1



Scheme 2



Scheme 3



CONDITIONS: **i:** 1N-HCl, 95 °C, 62%; **ii:** Ac_2O, Et_3N, DMF, 0 °C, 95%; **iii:** K_2CO_3, MeOH, 40 °C, 99%; **iv:** MeI (8eq.), Et_3N, DMF, 40 °C, 86% (for **5a**); CH_2ClBr (2.3eq.), Cs_2CO_3, DMF, 100 °C, 68% (for **5b**); $Br(CH_2)_2Br$ (6eq.), K_2CO_3, DMF, 110 °C, 85% (for **5c**); or CH_2ClBr (1.1eq.), Cs_2CO_3, DMF, 100 °C, 22% (for **5d**); **v:** MeI, K_2CO_3, DMF, 50 °C, 32%; **vi:** BnBr, K_2CO_3, DMF, 70 °C, 52%; **vii:** 6N-NaOH, $MeOCH_2CH_2OH$, reflux, 50-99%; **viii:** H_2, Pd/C, MeOH, rt., 89%; **ix:** RX, Et_3N, 0-80 °C, $CHCl_3$ or DMF, 37-99% (for **7a-d**, **7j**, **7r**, **7t**); R'COCl, Et_3N, CH_2Cl_2 or $CHCl_3$, 0 °C, 74-98% (for **7f-i**, **7k**, **7m**, **7o**); HCO_2H, WSC, DMAP, DMF, rt., 85% (for **7e**); EtOCOCl, Et_3N, CH_2Cl_2, 0 °C, 82%(for **7l**); or CH_3NCO, $CHCl_3$, rt., 86%(for **7q**); **x:** $LiBH_4$, THF, 0 °C, 37-53%; **xi:** H_2NNH_2, EtOH, rt., 59-71%.

using $LiBH_4$ in tetrahydrofuran respectively gave **7n** and **7p**. Formamide **7e** was prepared by the condensation of **6b** with formic acid using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (WSC) and dimethylaminopyridine in DMF. Ethyl carbamate **7l** was prepared by treating **6b** with ethyl chloroformate. Methyl urea **7q** was prepared by treating **6b** with methyl isocyanate.

Results and Discussion: The inhibitory activities of pavine derivatives with various substitutions on their aromatic rings are given in Table 1. In these series, amino substituents were fixed to the acetyl or the hydrogen. In the acetyl series, the dimethylenedioxy compound **5b** was the most active . Tetramethoxy (**5a**) and diethylenedioxy (**5c**) analogs showed slightly less inhibitory activity, and the dicatechol (**4**) and tetraacetoxy (**3**) analogs showed no activity The monomethylenedioxy analogs **5e** and **5d** had increased activity as compared with the corresponding tetramethoxy (**5a**) and dicatechol (**4**) analogs. This suggests that substitutions on aromatic rings preferred the lower electron donating function because the methylenedioxy group has an

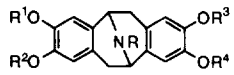


Table 1 ***In vitro*** **TNF-α inhibitory activity**[a]

Compound[b]	R	R^1	R^2	R^3	R^4	IC_{50} (μM)[d]	Compound[b]	R	R^1	R^2	R^3	R^4	IC_{50} (μM)[d]
4	Ac	H	H	H	H	>100	**2**[c]	H	H	H	H	H	>100
3	Ac	Ac	Ac	Ac	Ac	>100							
5a	Ac	Me	Me	Me	Me	38	**6a**[c]	H	Me	Me	Me	Me	>100
5b	Ac	$-CH_2-$		$-CH_2-$		13	**6b**[c]	H	$-CH_2-$		$-CH_2-$		15
5c	Ac	$-CH_2CH_2-$		$-CH_2CH_2-$		34	**6c**[c]	H	$-CH_2CH_2-$		$-CH_2CH_2-$		22
5e	Ac	$-CH_2-$		Me	Me	22	**6e**[c]	H	$-CH_2-$		Me	Me	100
5d	Ac	$-CH_2-$		H	H	46	**6d**[c]	H	$-CH_2-$		H	H	44
PTX[e]						68							

[a]Thioglycollate-elicited peritoneal macrophages obtained from Balb/c mice, as described by Kunkel et al.[9] were cultivated in MEM supplemented with 10% fetal bovine serum (FBS, Filtron, Victoria, Australia) in a 96 well culture plate. TNF-α was induced by stimulating 2 x 10^5 cells with 10 μg/ml of LPS (E.coli O111B4, DIFCO, Detroit, MI, USA) then culturing them for 18 h at 37°C. Compounds were dissolved in DMSO and added to the culture simultaneously with LPS. The final DMSO concentration was below 0.1%. The TNF-α concentration in the supernatant was measured by the ELISA. Briefly, the supernatant and recombinant mouse TNF-α standard (rm-TNF-α, Genzyme, Cambridge, MA, USA) were incubated overnight at 4°C in a 96 well plate coated with anti-mouse TNF-α monoclonal antibody (PharMingen, San Diego, CA, USA) that previously had been blocked with 10% FBS. Each well of the plate first was incubated at rt with biotinylated anti-mouse TNF-α polyclonal antibodies (PharMingen) for 45 min, then with peroxidase-conjugated streptavidin (Kirkegaard & Perry Laboratories Inc. , Gaitherburg, MD, USA) for 30 min. After each incubation, the plate was washed 4 to 8 times with PBS containing 0.2% Tween 20. TMB (Kirkegaard & Perry Laboratories Inc.) was the peroxidase substrate used, and the absorbance of each well was quantified at 450 nm by a microplatereader (Molecular Devices Corp., CA, USA). TNF-α levels in the supernatants were quantified with rm-TNF-α as the standard.

[b]All the compounds tested were racemic.

[c]Compound tested as the hydrochloride.

[d]Results are average of two independent experiments.

[e]Pentoxifylline

electronic contribution that is less than that of the other hydroxyl, methoxy and ethylenedioxy groups.[10] The secondary amino series (R = H) also had the activities similar to those of the acetyl derivatives, except for **6a** and **6e** which had less potency. We found that the dimethylenedioxy group on the aromatic rings gave the best results. Therefore we focused on substitutions on the amino nitrogen atom (Table 2). There was no marked difference in activity between the alkyl (**6b**, **7a-d**) and acyl (**5b**, **7e-i**) derivatives; but, increasing the size of the substituents led to diminished potency in both series. Taking into account the steric limitation of a substituent in this region, the effects of other functions (**7j-q**, **7s**, **7u**) were investigated. These compounds retained inhibitory activity.

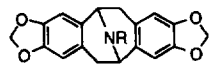


Table 2 ***In vitro*** **TNF-α inhibitory activity**[a]

Compound[b]	R	IC_{50} (μM)[d]	Compound[b]	R	IC_{50} (μM)[d]
6b[c]	H	15	**7j**[c]	$(CH_2)_2OH$	36
7a[c]	Me	19	**7k**	$COCH_2OCH_3$	17
7b[c]	Et	27	**7l**	CO_2Et	31
7c[c]	n-Pr	56	**7m**	$COCO_2Et$	15
7d[c]	n-Bu	58	**7o**	$COCH_2CO_2Et$	26
7e	CHO	22	**7n**	$COCH_2OH$	20
5b	Ac	13	**7p**	$CO(CH_2)_2OH$	27
7f	$COCH_2CH_3$	11	**7q**	CONHMe	15
7g	$CO(CH_2)_2CH_3$	11	**7s**[c]	$(CH_2)_2NH_2$	14
7h	$CO(CH_2)_4CH_3$	>100	**7u**[c]	$(CH_2)_3NH_2$	52
7i	Bz	38			

[a]See footnotes to Table 1

[b]All the compounds tested were racemic.

[c]Compound tested as the hydrochloride.

[d]Results are averages of two independent experiments.

To demonstrate the usefulness of these derivatives *in vivo*, we evaluated several compounds orally for protection against LPS-induced death of galactosamine-sensitized mice (Table 3). The administration of LPS to mice induces a typical death causing shock, and it has been suggested that TNF acts as a major mediator for the development of the disease in this model.[1, 2a,b, 11] Of these compounds, **5b**, **6b**, **7b**, **7e**, **7f**, **7j**, and **7s** had high potency (more than 70% survival rate at 50 mg/kg). *In vitro* and *in vivo* activities were correlated, except for a few compounds (i.e. **6c**, **7g**, **7m**, **7o**) which were less potent than expected for *in vitro* activity. This could be due to differences in the pharmacokinetics profile; i.e. in administration, distribution, metabolism, and excretion. Pentoxifylline (PTX, SIGMA Co.) was the positive control used to evaluate our compounds.[12] We found that most of the compounds prepared were more potent than PTX in both the *in vitro* and *in vivo* tests.

Table 3 Protection against LPS-induced death of D-galactosamine-sensitized mice[a]

	Survival rate, day 7				Survival rate, day 7		
Compound[b]	0	50	100(mg/kg)	Compound[b]	0	50	100(mg/kg)
5b	0/10	7/10	9/10	**7j**[c]	0/10	7/10	9/10
5c	0/10	4/10	7/10	**7l**	0/10	3/10	3/10
6b[c]	0/10	7/10	10/10	**7m**	0/10	3/10	2/10
6c[c]	0/10	1/10	4/10	**7n**	0/10	6/10	9/10
7b[c]	0/10	9/10	10/10	**7o**	0/10	2/10	3/10
7e	0/10	7/10	7/10	**7p**	0/10	5/10	6/10
7f	0/10	8/10	8/10	**7q**	0/10	6/10	6/10
7g	0/10	4/10	5/10	**7s**[c]	0/10	8/10	8/10
PTX[d]	0/10	N.T.	4/10				

[a]Female BALB/c mice (5 weeks old) were injected i.v. with a mixture of D-galactosamine (1g/kg) and LPS (1μg/kg). More than 80% of the mice died of endotoxin-shock within 48h. Compounds were dissolved in 10% NIKKOL® solution (Nippon Surfactant Kougyou, Japan) containing 5% DMSO and administered orally to mice immediately after D-galactosamine/LPS injection. The survival rates of the 10 mice in each treatment were monitored during the last 7 days of the experiment.

[b]All the compounds tested were racemic.

[c]Compound tested as the hydrochloride.

[d]Pentoxifylline

[e]Not tested

In conclusion, the substitution on the aromatic rings of pavine structure was important for inhibitory activity, and the dimethylenedioxy group gave good results. Substitutions on nitrogen atom reduced activity as the size of the substituent increased, indicative of steric limitation in this region. These derivatives also had potent inhibitory activity *in vivo*. Efforts to improve the TNF inhibitory activities of the compounds in this series are underway.

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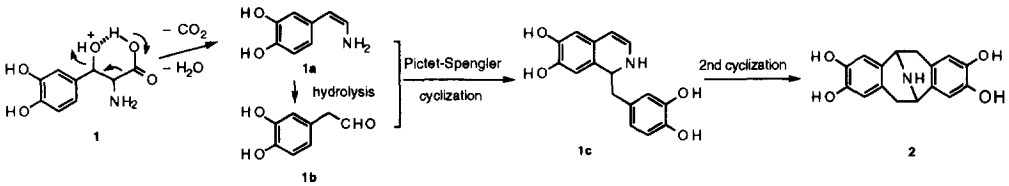
5. We speculate that the mechanism of this reaction is as follows: Decarboxylation and dehydration of **1** gave the enamine **1a** which was easily hydrolyzed to give aldehyde **1b**. Subsequent Pictet-Spengler cyclization[5a] (**1c**) and intramolucular cyclization[5b, 5c] gave **2**. Experimental clarification of this mechanism is underway.



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