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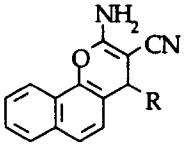
THE ANTI-RHEUMATIC POTENTIAL OF A SERIES OF 2,4-DI-SUBSTITUTED-4H-NAPHTHO[1,2-b]PYRAN-3-CARBONITRILES.

Colin W. Smith, James M. Bailey, Michael E. J. Billingham[a], Srinivasan Chandrasekhar[b], Colin P. Dell, Anita K. Harvey[b], Caroline A. Hicks, Ann E. Kingston and Graham N. Wishart.*
Lilly Research Centre Ltd., Eli Lilly & Company, Windlesham, Surrey GU20 6PH, U. K.

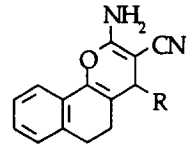
Abstract: A new series of naphtho[1,2-b]pyran-3-carbonitriles with enhanced stability under acid conditions has been synthesised and examined for antiproliferative and anti-inflammatory activity. 4-(3-nitrophenyl)-2-(N-succinimido)-4H-naphtho[1,2-b]pyran-3-carbonitrile, **10**, has proved to be acid stable and still retains biological activity.

In a recent publication, results were disclosed which were derived from testing a series of 4-substituted-4H-naphtho[1,2-b]pyrans (and appropriate 5,6-dihydro-analogues) on a neutral metalloprotease activity assay (NMPA)[1]. This series represents the only group of compounds that these authors found, (other than glucocorticoids) that blocked the synthesis of metalloproteases, enzymes that are responsible for destruction of articular cartilage in diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA)[2]. Interestingly, the lead compound **1a** inhibits the synthesis of collagenase and stromelysin at protein and mRNA levels, yet shows no direct inhibition of the active enzymes[1]. The most potent examples (Table 1) were compounds containing 4-(3-substituted-phenyl)-groups, as exemplified by the 3-nitro compound **1a** and its 5,6-dihydro-counterpart **2** (Scheme 1). We have synthesised some additional examples also by the method described by Elagamey *et al.*[3] (**1b** and **1c**, Scheme 1), examples **1b** and **1c** show that compounds bearing a dihaloaryl or heteroaryl moiety respectively, can still maintain potency in this series.

Scheme 1



1a, R = 3-nitrophenyl
1b, R = 3-chloro-4-fluoro
1c, R = 3-pyridyl



2, R = 3-nitrophenyl

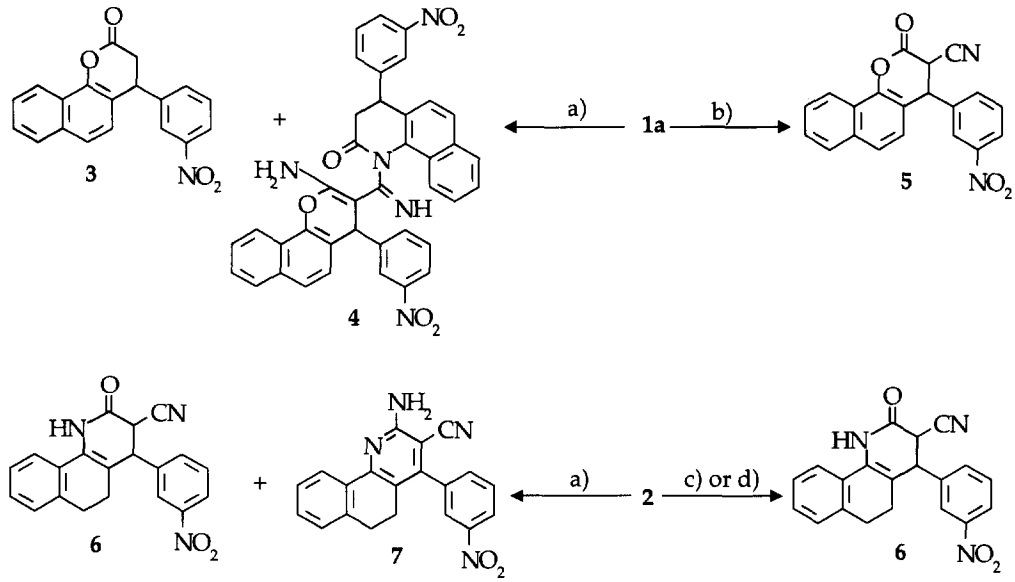
We have found that these compounds also inhibit spleen cell proliferation induced by the mitogen concanavalin A (Con A)[4]. Results have been obtained using rat and mouse spleen cells. Data on the mouse cells were found to be very similar when compared with the rat data and are not included here. Inhibition of proliferation is detected at submicromolar concentrations, this is impressively demonstrated by compounds **1a**, **1b**, **1c** and **2**, (Table 2).

Investigation of the therapeutic potential of these compounds *in vivo* included absorption, distribution, metabolism and excretion (ADME) studies. A [^{14}C]labelled analogue (at C-2) of compound **1a** was selected for preliminary studies and was administered intravenously at 5mg/kg to F-344 male rats. Parent compound was rapidly cleared with a half life of approximately 1 h, but only accounted for about 16% of the circulating radioactivity. Extensive first pass metabolism was demonstrated, with elimination of a number of unidentified metabolites *via* the bile. In addition to rapid metabolism, instability of **1a** and other members of the series in acid solution (50/50/0.1,

v/v/v/acetonitrile/water/TFA) was apparent during development of HPLC analytical methods. These compounds formed several degradation products which were difficult to identify.

Because of the instability of these compounds it was decided to attempt to synthesise acid stable analogues with a view to finding candidates with *in vivo* stability which could be used in the treatment of rheumatic diseases. However it was desirable, initially, to ascertain whether the *in vitro* activity of the series might be due to the presence of degradation products. Therefore compounds **1a** and **2** were subjected to a variety of acid conditions (Scheme 2), and products that could be isolated were tested on the NMPA and Con A proliferation assays (Tables 1 and 2). The minor and structurally distinct component **4** and compound **5** were not tested.

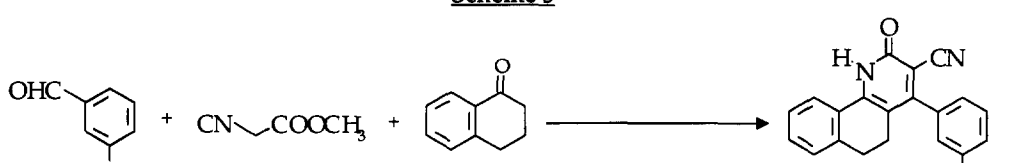
Scheme 2



Reagents: a) NH_4OAc, AcOH, reflux
b) 1M HCl, DMSO, 50°C
c) AcOH, reflux
d) HCOOH, HCl(g), THF, 25°C

The 3,4-dihydronaphtho[1,2-b]pyran-2(1H)-one **3**, the 3,4,5,6-tetrahydrobenzo[h]quinolin-2(1H)-one **6** (a Dimroth rearrangement product[5]) and the 5,6-dihydrobenzo[h]quinoline-3-carbonitrile **7**, (after separation from **6**) could be isolated and were tested on the two assays. A known compound **8**[6], which is structurally similar to **6**, was made, albeit by a different synthesis[7], (Scheme 3) and was also tested on both assays. None of these four compounds was particularly active, and it is reasonable to assume that the parent naphtho[1,2-b]pyrans are the active species.

Scheme 3



Reagents: NH_4OAc, n-butanol, reflux

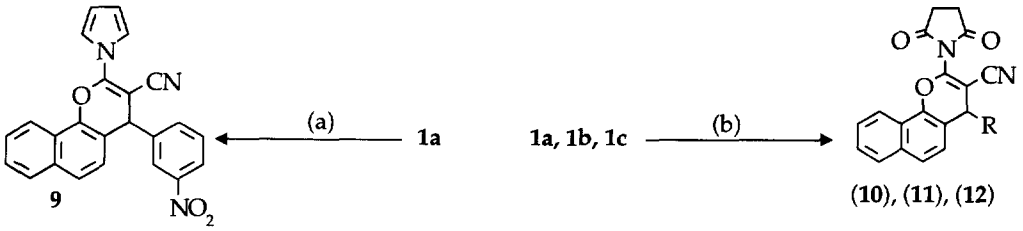
Table 1: Percentage Inhibition of Neutral Metalloprotease Activity.

	Conc./(μM)			
Compound No.	**10**	**1**	**0.1**	**mp / ^{o}C**
1a	102	76	74	215-216
1b	81^{T}	111	37	211-212
1c	100	83	75	205-207
2	104	76	39	175-176
3	ND	ND	ND	190-191
5	ND	ND	ND	240-248
6	20	36	-7	216-219
7	42	36	8	199-201
8	36	3	36	>300 lit.[6]
9	4	-5	1	198-199[8]
10	86	69	17	230-231
11	111	53	26	222-223
12	25	17	-17	249-250

Description of NMPA determinations: Rabbit chondrocytes were grown to confluency in 96 well microtitre plates. The media were removed and the cells were treated with serum-free macrophage conditioned medium (MCM) and the compounds. The media were assayed for NMPA, all determinations were the average of two experiments and were performed in quadruplicate. They were considered to be toxic (T) if cell viability was less than 90% as determined by a trypan blue exclusion test. The inhibitory effects of the compounds were calculated as: % inhibition = 100[NMP in MCM/NMP in the test], inhibitions were given as +ve values. The results were all within 10% of the average values shown. ND = not determined. For full experimental procedures see reference 1.

Derivatives of the naphthopyrans which are stable at physiological pH might have a measurable concentration in plasma. If such derivatives are active *in vitro* and if they are absorbed, they may be active in an appropriate *in vivo* assay. Since it is the enamine moiety of these compounds that makes them vulnerable to acid attack modification of the amino group was thought to be the best way of stabilising them whilst still retaining activity. A pyrrolo derivative of **1a**, compound **9** and succinimido derivatives of compounds **1a**, **1b** and **1c** (**10**, **11** and **12** respectively) were prepared by literature methods[9,10], (Scheme 4), and tested on both assays.

Scheme 4



9 **(10), (11), (12)**

Reagents: (a) AcOH, 2,5-dimethoxytetrahydrofuran, reflux
(b) DMF, N,N-carbonyldiimidazole, succinic acid, 80-90^{o}C

Table 2: Percentage Inhibition of Concanavalin A Stimulated Rat Spleen Cell Proliferation

	Conc./ (μM)				
Compound No.	**10**	**1**	**0.1**	**0.01**	**0.001**
1a	99	95	89	73	33
1b	98	86	83	38	ND
1c	ND	95	89	11	0
2	99	95	87	58	29
3	15	2	9	6	19
5	ND	ND	ND	ND	ND
6	22	15	6	8	9
7	46	23	23	15	13
8	ND	ND	ND	ND	ND
9	87	82	-7	ND	ND
10	97	87	-4	1	9
11	98	92	10	-16	18
12	87	85	-5	-6	7

Description of the Con A Proliferation assay: Female Lewis rats, weighing 250 g ± 50 g, were used to provide splenic lymphocytes as described in ref. 4. The cells were cultured in 96 well microtitre plates at a density of 10^5 cells per well. Compounds were added to the cultures before addition of Con A (2mg/mL) to give a final volume of 200mL per well. Cultures were incubated at 37°C in a humidified 5% CO_2 in air atmosphere for 72 h. [^{3}H]thymidine was added to cultures (1mCi/well) 6h before harvesting the cells using a Skatron automatic harvester. [^{3}H]thymidine incorporation was determined by liquid scintillation counting.
Percentage inhibition of the Con A response was calculated according to the equation:
% Inhibition = 100[1-(cpm culture+compound/cpm culture control)]. All determinations represent the mean of quadruplicate cultures and the results are representative of at least two experiments.

Although compound **9** was active on the Con A proliferation assay, it was inactive on the NMPA assay and was not considered further. The succinimides **10, 11** and **12** showed marked inhibition on the Con A assay at 1μM concentrations, and it was also evident that compounds **10** and **11** were active on the NMPA assay at submicromolar concentrations. Gratifyingly when exposed to the acid conditions (described earlier) all three succinimides (**10, 11** and **12**) were stable. Furthermore some oral bioavailability could be demonstrated when these compounds were administered to male guinea pigs at 25 mg/kg body weight by gavage. Plasma concentrations were measured over 0 to 8 h and model independent pharmacokinetic parameters were estimated (Table 3) which showed increasing concentrations of circulating parent-succinimide.

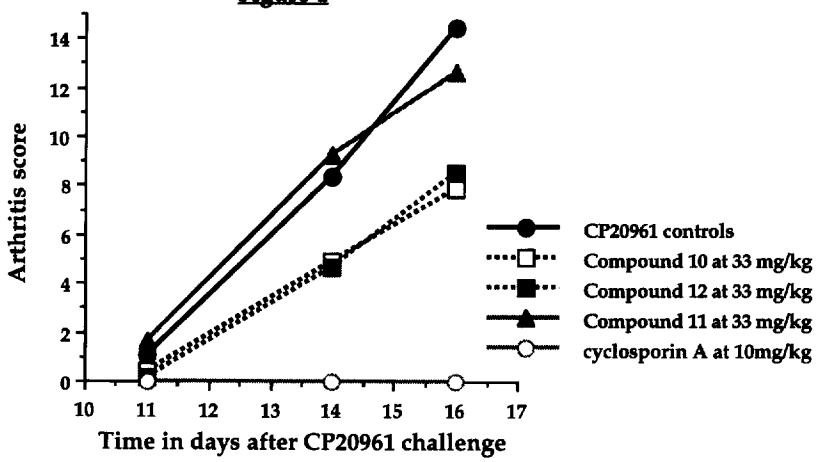
Table 3: Model Independent Pharmacokinetic Parameters for the 3 succinimides

Compound No.	AUC (0-8 h, mg/mL)	Cmax (ng/mL)	Tmax (h)
10	ND	5	6
11	0.34	178	4
12	0.34	55	0.5

AUC = area under the curve, Tmax is when the maximum concentration is achieved and Cmax is the concentration at Tmax.

A convincing *in vivo* model that can be directly related to the metalloprotease inhibitory activity of succinimides **10, 11** and **12** is not currently available. However the *in vivo* activity of these compounds can be studied using an experimental adjuvant arthritis model of RA since the Con A assay is an excellent *in vitro* assay for predicting positive activity in this model, because of the involvement of proliferating T-lymphocytes in the disease process. Therefore succinimides **10, 11** and **12**, which were active on the Con A assay, were subjected to a version of the developing adjuvant arthritis model where the arthritis was induced by the synthetic lipoidal amine CP20961[11]. The ensuing disease was assessed by a visual scoring method in preference to conventional paw volume measurements[12,13].

Figure 1



Description of the adjuvant arthritis model: Arthritis was induced by injecting intradermally CP20961 (5 mg in 0.1 mL of warmed paraffin oil) into the tail base of female inbred Lewis rats (OLAC, U.K.). 10 animals per group, each weighing 140-170g at the beginning of each experiment were used. The arthritis was measured using a visual scoring method based on the severity at eight lesion sites, at 2-3 day intervals, these were the hind and forepaws, the tail, ears, eyes and nose. On a scale of 0 = no signs of arthritis and 4 = severe arthritis a total score of 32 was achievable, but in practice experiments were stopped before this stage was reached. Cyclosporin A was used as a positive standard.

Compound **10**, which was active on both *in vitro* assays, showed a 48% reduction in the arthritis score by day 16 (Figure 1). Compound **12** showed a 41% reduction in the arthritis score by day 16 (this was active on the Con A assay but not very active on the NMPA assay). Compound **11** showed no reduction in arthritis score even though it was active on the Con A assay. Cyclosporin A showed a 100% reduction in the arthritis score from day 11 to day 16.

Summary:

We have demonstrated that the potent *in vitro* activity of a novel series of naphtho[1,2-b]pyrans, described in an earlier publication[1], is not retained in degradation products isolated after two representative compounds were exposed to acid conditions. The *in vitro* activity is however

retained in a small series of bioavailable acid-stable succinimido-derivatives, two examples of which reduced the inflammatory response in a developing adjuvant model. Only the anti-arthritic potential of these compounds has been addressed in this communication. Currently there is a lot of interest in the literature regarding metalloprotease inhibitors which indicate that they possess considerable potential for the treatment of a number of medical conditions, including arthritis[14]. Stable naphtho[1,2-b]pyran derivatives which inhibit the synthesis of metalloproteases, yet are not direct enzyme inhibitors, could be an important addition to this list of interesting agents.

[a]Address: Rheumatology Unit, Dept. of Medicine, Bristol Royal Infirmary, Bristol BS2 8HW, U.K.
[b]Address: Endocrine Division, Lilly Research Labs., Corporate Center, Indianapolis, IN 46285, U.S.A.

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