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(54) Title: ANTI-INFLAMMATORY COMPOSITION

(57) **Abrégé/Abstract:**

The invention relates to 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2- one, and its pharmaceutical compositions and its use for preparing a medicament intended to prevent or treat inflammatory disorders.



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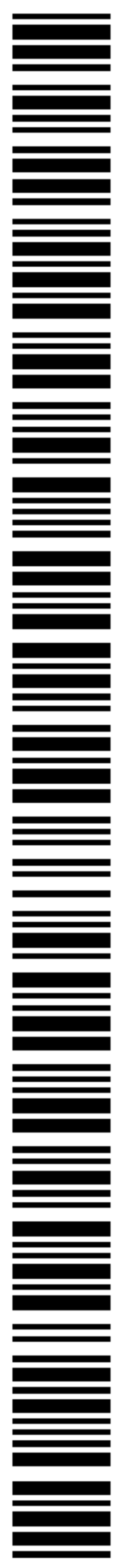
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(54) Title: ANTI-INFLAMMATORY COMPOSITION

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Anti-inflammatory Composition

The invention relates to 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one, and its pharmaceutical compositions and its use for preparing a medicament intended to prevent or treat inflammatory disorders.

5 Inflammation is an important component of physiological host defence. Increasingly, however, it is clear that temporally or spatially inappropriate inflammatory responses play a part in a wide range of diseases, including those with an obvious leukocyte component (such as autoimmune diseases, asthma or atherosclerosis) but also in
10 osteoporosis or Alzheimer's disease).

The chemokines are a large family of signalling molecules with homology to interleukin-8, which have been implicated in regulating leukocyte trafficking both in physiological and pathological conditions. With more than fifty ligands and twenty receptors involved in chemokine signalling, the system has the requisite information
15 density to address leukocytes through the complex immune regulatory processes from the bone marrow, to the periphery, then back through secondary lymphoid organs. However, this complexity of the chemokine system has at first hindered pharmacological approaches to modulating inflammatory responses through chemokine receptor blockade. It has proved difficult to determine which chemokine
20 receptor(s) should be inhibited to produce therapeutic benefit in a given inflammatory disease.

More recently, a family of agents which block signalling by a wide range of chemokines simultaneously has been described: Reckless et al., Biochem J. (1999) 340:803-811. The first such agent, a peptide termed "Peptide 3", was found to inhibit
25 leukocyte migration induced by 5 different chemokines, while leaving migration in response to other chemoattractants (such as fMLP or TGF-beta) unaltered. This peptide, and its analogs such as NR58-3.14.3 (i.e. Sequence ID No.1 c(DCys-DGln-DIle-DTrp-DLys-DGln-DLys-DPro-DAsp-DLeu-DCys)-NH₂), are collectively termed "Broad Spectrum Chemokine Inhibitors" (BSCIs). Grainger et al., Biochem.
30 Pharm. 65 (2003) 1027-1034 have subsequently shown BSCIs to have potentially

useful anti-inflammatory activity in a range of animal models of diseases.

Interestingly, simultaneous blockade of multiple chemokines is not apparently associated with acute or chronic toxicity, suggesting this approach may be a useful strategy for developing new anti-inflammatory medications with similar benefits to
5 steroids but with reduced side-effects.

However, peptides and peptoid derivatives such as NR58-3.14.3, may not be optimal for use in vivo. They are quite expensive to synthesise and have relatively unfavourable pharmacokinetic and pharmacodynamic properties. For example, NR58-3.14.3 is not orally bioavailable and is cleared from blood plasma with a half-
10 life period of less than 30 minutes after intravenous injection.

Two parallel strategies have been adopted to identify novel preparations that retain the anti-inflammatory properties of peptide 3 and NR58-3.14.3, but have improved characteristics for use as pharmaceuticals. Firstly, a series of peptide analogs have been developed, some of which have longer plasma half-lives than NR58-3.14.3 and
15 which are considerably cheaper to synthesise. Secondly, a structure: activity analysis of the peptides has been carried out to identify pharmacophores in order to propose small non-peptidic structures which might retain the beneficial properties of the original peptide.

This second approach yielded several structurally distinct series of compounds that
20 retained the anti-inflammatory properties of the peptides, including 16-amino and 16-aminoalkyl derivatives of the alkaloid yohimbine, as well as a range of N-substituted 3-aminoglutarimides. (Reference: Fox et al., J Med Chem 45(2002) 360-370; WO 99/12968 and WO 00/42071). All of these compounds are broad-spectrum chemokine inhibitors which retain selectivity over non-chemokine chemoattractants,
25 and a number of them have been shown to block acute inflammation in vivo.

The most potent and selective of these compounds was (S)-3-(undec-10-enoyl)-aminoglutarimide (NR58,4), which inhibited chemokine-induced migration in vitro with an ED₅₀ of 5nM. However, further studies revealed that the aminoglutarimide ring was susceptible to enzymatic ring opening in serum. Consequently, for some
30 applications (for example, where the inflammation under treatment is chronic, such as

in autoimmune diseases) these compounds may not have optimal properties, and a more stable compound with similar anti-inflammatory properties may be superior.

As an approach to identifying such stable analogs, various derivatives of (S)-3-(undec-10-enoyl)-aminoglutarimide have been tested for their stability in serum. One such derivative, the 6-deoxo analog (S)-3-(undec-10-enoyl)-tetrahydropyridin-2-one,
5 is completely stable in human serum for at least 7 days at 37°C, but has considerably reduced potency compared with the parental molecule.

One such family of stable, broad spectrum chemokine inhibitors (BSCIs) are the 3-amino caprolactams, with a seven-membered monolactam ring (see, for example,
10 WO2005/053702 and WO2006/016152). However, further useful anti-inflammatory compounds have also been generated from other 3-aminolactams with different ring size (see for example WO2006/134385). Other modifications to the lactam ring, including introduction of heteroatoms and bicyclolactam ring systems, also yield compounds with BSCI activity (see, for example, WO2006/018609 and
15 WO2006/085096).

To date, the identification of broad classes of agents with BSCI activity, and hence anti-inflammatory properties in vivo, has been based on optimising potency of the BSCI activity. For example, previous disclosures taught that introduction of 2,2-disubstitution (at the alpha- or key-carbon atom in the acyl side chain of acyl-3-aminolactams) leads to a considerable increase in potency as a BSCI, both in vitro and
20 in vivo in models of acute inflammation, whether the 2,2-disubstituted acyl group was open chain (see WO2005/053702), monocyclic (see WO2006/134384) or polycyclic (see WO2006/016152).

However, potency of the desired pharmacological effect is only one factor in
25 determining whether an agent will make a useful human pharmaceutical, albeit an important factor. In particular, the pharmacokinetics (or disposition of the agent within the body) exerts a major effect on the utility of a particular agent.

Pharmacokinetics (defined in its broadest sense, as the study of the effects of the body on the drug, in contrast to pharmacodynamics, which is the study of the effects of the
30 drug on the body) depends on a host of complex physiological processes, including

(but not limited to) absorption, plasma stability, volume of distribution (and in particular rate of equilibration into 'target' tissues), metabolic transformation (including hepatic metabolism, such as cytochrome P450 isoenzyme mediated oxidation, and phase II metabolism such as sulfation and glucuronidation, and
5 extrahepatic metabolism, such as serum enzymic modification), and excretion (such as renal clearance into urine and fecal elimination). These processes are often collectively referred to as the 'ADME' properties of the agent (ADME being an acronym for Absorption, Distribution, Metabolism and Excretion).

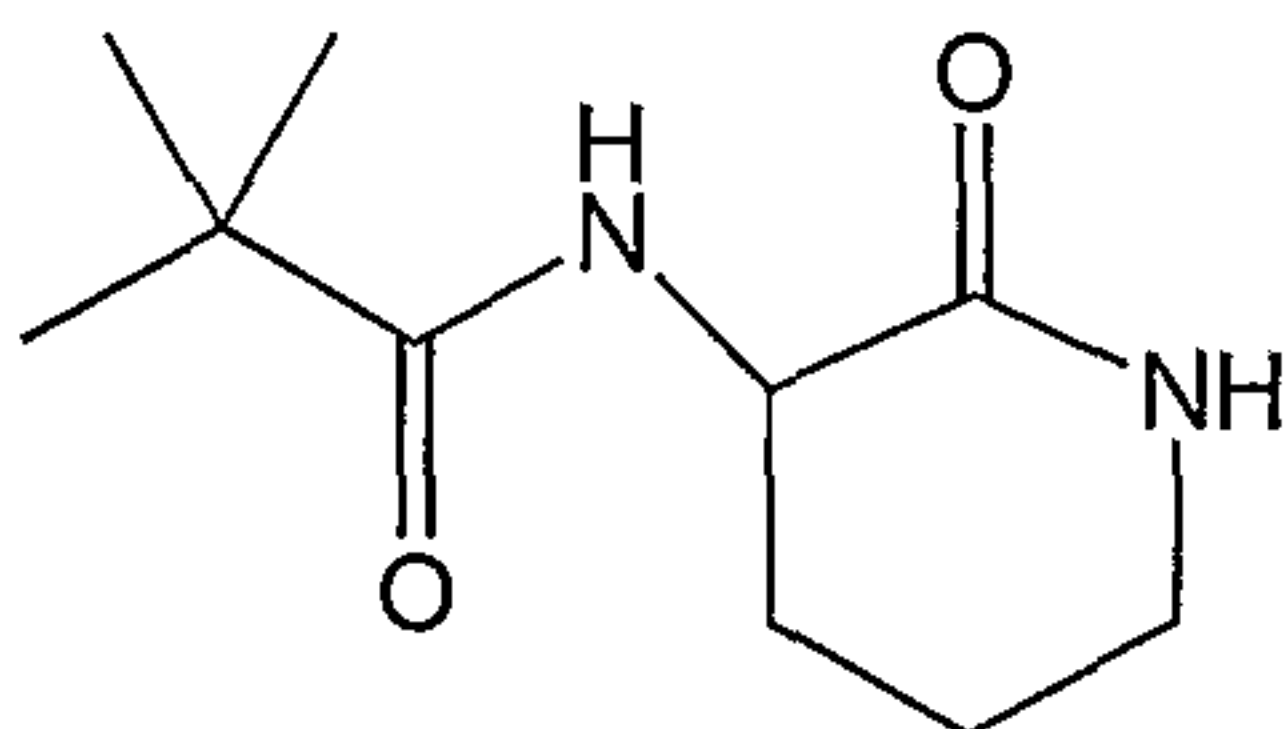
Another important factor in determining the utility of an agent as a human
10 pharmaceutical is safety. Many, if not all, compounds administered elicit multiple effects on the body of which the desirable pharmacological effects are usually only a subset. The remaining effects may result in harm (toxic effects) or inconvenience (side-effects) to the patient. The study of such properties of candidate pharmaceutical agents is called toxicology or safety pharmacology. Unwanted effects can be broadly
15 classified into two types. *Class effects* are intimately tied up with the desired pharmacological action, and (to a greater or less extent) are an inevitable consequence of manipulating the chosen molecular target. For example agents designed to prevent pathological inflammation may, to a degree, result in immunosuppression and an increased risk of infection. This is because inflammatory tissue damage and infection
20 are both inextricably linked to the degree of immune system activity. As a result, all molecules sharing the identical pharmacological target will, to a greater or lesser extent, share class effects. In contrast, *compound effects* are specifically associated with a particular compound structure, usually as a result of an (often unexpected) interaction with a target distinct from the intended pharmacological target. In
25 principle, it is possible to find another molecule with the same intended pharmacological effects but which is completely devoid of the compound-specific side-effects. Some compound effects are common (such as hERG interaction, which can result in dangerous prolongation of the QT interval during heart pacing, resulting potentially fatal cardiac arrhythmias), while other compound effects may be apparently
30 unique to the particular compound.

Crucially, despite decades of pharmaceutical development experience, there is still no generally accepted method for predicting either the ADME and pharmacokinetic properties of an agent, or its toxicology and safety pharmacology. It is for this reason that explicit testing, first using in vitro assay systems (such as hERG-expressing cell
5 lines), then in animals and finally in phase I clinical trials in man, is a regulatory requirement worldwide for the development of a new pharmaceutical.

Methods have been described for predicting certain aspects of ADME from inspection of the molecular structure, and there can be little doubt that experienced medicinal chemists can reliably rule out many structures on purely theoretical grounds. An
10 example of such a “rule of thumb” (for it is no more dependable than that) would be Lipinsky’s “Rules of Five”, based on the observation that most approved pharmaceuticals meet certain criteria related to molecular weight, number of rotatable bonds and polarity. Similarly, it is generally well known that molecules with large, hydrophobic groups are more likely to show an undesirable interaction with the hERG
15 channel.

Such general guidelines, even when applied together, may be useful for eliminating unsuitable molecules but many very unsuitable molecules (for various reasons) would still slip through the net. Today, no-one would seriously countenance selecting a drug candidate from a class of active compounds on purely theoretical grounds. As a
20 result, the discovery of a particular compound from within a class which has particularly advantageous ADME, pharmacokinetic, toxicological and safety pharmacological properties requires considerable practical experimentation among good candidates, and is a novel finding which could not be predicted even by those skilled in the art.

25 Here we describe the novel compound 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one (I), which has not previously been reported.

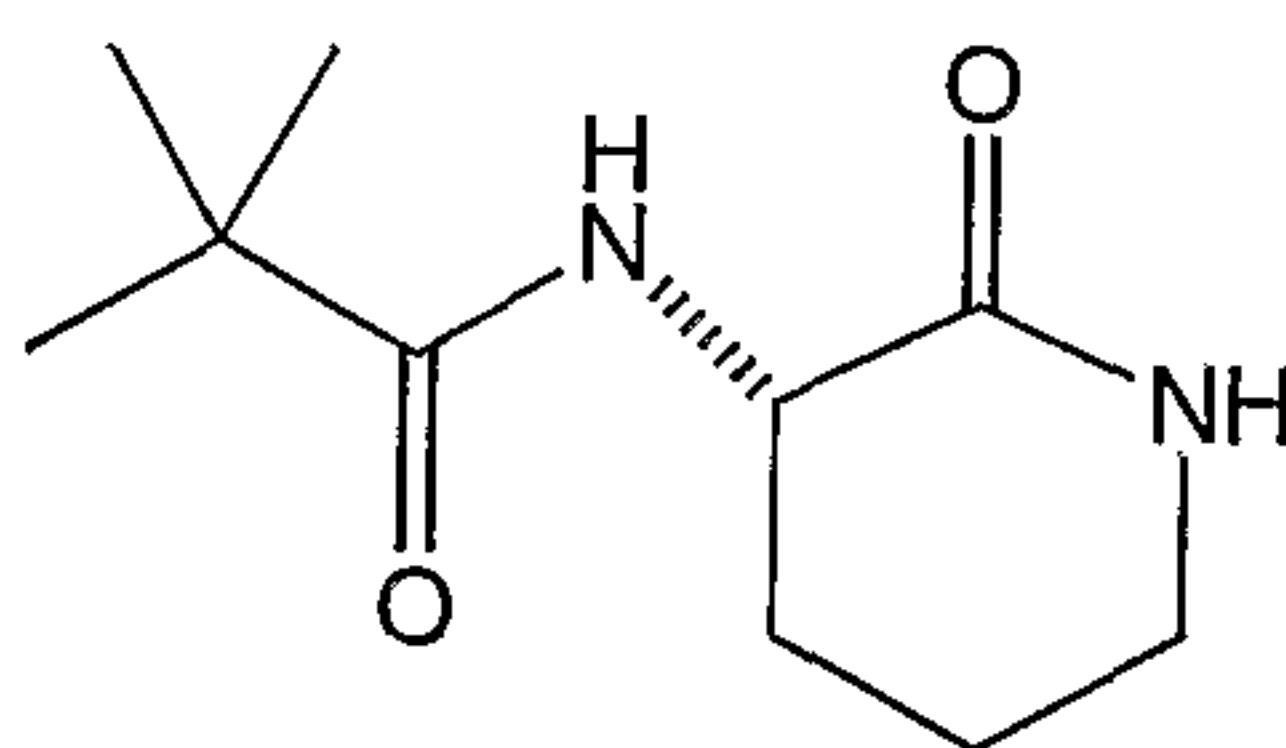


(I)

This compound is a specific member of the broad generic class of BSCIs which have been described previously (for example, see WO2006/134385). However, we now demonstrate that while all the molecules of the class have BSCI activity, compounds (I) has significantly superior properties for use as a human pharmaceutical as a result of its combination of ADME, pharmacokinetic, toxicology and safety pharmacology properties, when compared experimentally to other members of the class.

The carbon atom at position 3 of the lactam ring is asymmetric and consequently, the compounds according to the present invention have at least two possible individual forms, that is, the "R" and "S" configurations. The present invention encompasses the two enantiomeric forms and all combinations of these forms, including the racemic "RS" mixtures. With a view to simplicity, when no specific configuration is shown in the structural formula, it should be understood that the two individual enantiomeric forms and their mixtures are represented. Since enantiomeric inversion has no effect on the key ADME properties responsible for the superiority of the compound (and additionally has only a small effect on the potency of the compound as a BSCI), both enantiomeric forms, as well as their admixtures, represent specific examples which are materially superior to the class in general.

Preferably, the compound of formula (I) according to this invention will be the compound of formula (I').



(I')

The compound (I'), having the (S)-configuration at the stereocentre, is 5-25 fold more potent as a BSCI than the (R)-enantiomer.

Also provided are pharmaceutical compositions, comprising, as active ingredient, a compound of general formula (I) or (I'), or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable excipient and/or carrier.

By pharmaceutically acceptable salt is meant in particular the addition salts of
5 inorganic acids such as hydrochloride, hydrobromide, hydroiodide, sulphate, phosphate, diphosphate and nitrate or of organic acids such as acetate, maleate, fumarate, tartrate, succinate, citrate, lactate, methanesulphonate, p-toluenesulphonate, palmoate and stearate. Also within the scope of the present invention, when they can be used, are the salts formed from bases such as sodium or potassium hydroxide. For
10 other examples of pharmaceutically acceptable salts, reference can be made to "Salt selection for basic drugs", *Int. J. Pharm.* (1986), 33:201-217.

The pharmaceutical composition can be in the form of a solid, for example powders, granules, tablets, gelatin capsules, liposomes or suppositories. Appropriate solid supports can be, for example, calcium phosphate, magnesium stearate, talc, sugars,
15 lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidone and wax. Other appropriate pharmaceutically acceptable excipients and/or carriers will be known to those skilled in the art.

The pharmaceutical compositions according to the invention can also be presented in liquid form, for example, solutions, emulsions, suspensions or syrups. Appropriate
20 liquid supports can be, for example, water, organic solvents such as glycerol or glycols, as well as their mixtures, in varying proportions, in water.

The invention also provides the use of a compound of formula (I) or (I'), or a pharmaceutically acceptable salt thereof, for the preparation of a medicament intended to treat inflammatory disorder.

25 The invention includes compounds, compositions and uses thereof as defined, wherein the compound is in hydrated or solvated form.

In comparison to the prior art the improvement of the present invention lies in the unexpected observation that 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one has superior ADME properties compared to the general classes of lactam BSCIs

which have been described previously (such as, for example, the International applications *supra*). Although such compounds were reported as having acceptable pharmacodynamic properties (that is, they have a potent anti-inflammatory effect in vivo as a result of their BSCI activity), and it was inferred that they must have acceptable pharmacokinetic, and hence ADME, properties, nevertheless direct assessment of the ADME properties suggest that 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one is notably and unexpectedly superior (see the examples below).

In particular, while previous in vitro stability studies in serum suggested that lactam BSCIs were considerably better than the earlier imide BSCIs (see, for example, WO99/12968), as reported in the literature (for example Fox et al. *J. Med. Chem.* 2005 48:867-74), it is now clear that many (or indeed most) of the lactam class of BSCIs are subject to unwanted metabolism in vivo. We have made and tested more than a dozen BSCIs of the acylaminolactam class, and with the exception of 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one, all of the lactam class BSCIs tested to date are subject to rapid liver metabolism (either cytochrome P450 mediated hydroxylation and/or phase II metabolism).

At least partly as a consequence of the reduced in vivo metabolism, the overall clearance of 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one is markedly lower than for the other lactam BSCIs tested. As a result, the exposure following a single oral dose is more than 10-fold higher for 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one. 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one is therefore more suitable for use as a human pharmaceutical, particularly where chronic oral exposure is required for efficacy, than most (if not all) of the lactam BSCIs previously disclosed.

Prior art peptides (such as NR58-3.14.3) have the disadvantages that: (a) they are expensive and require solid phase synthesis (at least for the longer ones) and (b) they clear very quickly via the kidneys and (c) they are generally very much less potent (>25 fold less potent in vitro and >10,000 fold less potent in vivo).

The prior art aminoglutarimides are cheap, not cleared quickly via the kidneys and more potent in vitro BUT they are extremely unstable in serum (as a result of an enzymatic opening of the imide ring; see, for example, Fox et al. *J. Med. Chem.* 2005 48:867-74). As a result aminoglutarimide BSCIs, such as (S)-3-(undec-10-
5 enoylamino)glutarimide, are at least 250-fold less potent in vivo than 2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one, even in models of acute inflammation (such as LPS-induced endotoxemia marked by systemic TNF- α production, where the impact of compound stability and ADME properties is least apparent).

10 Another series of structurally related (but functionally largely dissimilar) compounds which have been described in the literature are bacterial autoinducer compounds typically based around the 6-membered homoserinelactone structure, usually with a 3-oxo-acyl side chain (for example, see Bycroft et al. US5,969,158 which claims a broad range of such compounds). Interestingly, although such disclosures include
15 generic formulae that encompass lactams as well as lactones, few if any of the exemplified compounds with bacterial autoinducer properties have lactam headgroups. All such compounds (but particularly those with a lactone headgroup and/or a 3-oxoacyl tail group) are known to be relatively unstable limiting their applications as medicaments.

20 The improvement described here, 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one, is cheap to synthesise (and the method disclosed here allows straightforward synthesis even on a Kg scale), and shows excellent metabolic stability not only in isolated serum in vitro (a property shared with the whole lactam class of BSCIs disclosed previously) but also in vivo. As a result, the compounds of the
25 present invention is (among those compounds studied extensively to date) uniquely optimised for the treatment of inflammatory diseases in man in terms of efficacy, potency and pharmaceutical properties such as ADME, pharmacokinetics, toxicology and safety pharmacology.

According to this invention, inflammatory disorders intended to be prevented or
30 treated by the compounds of formula (I) or (I') or the pharmaceutically acceptable

salts thereof or pharmaceutical compositions or medicaments containing them as active ingredients include notably:

- autoimmune diseases, for example such as multiple sclerosis, rheumatoid arthritis, lupus, irritable bowel syndrome, Crohn's disease;
- 5 - vascular disorders including stroke, coronary artery diseases, myocardial infarction, unstable angina pectoris, atherosclerosis or vasculitis, e. g., Behçet's syndrome, giant cell arteritis, polymyalgia rheumatica, Wegener's granulomatosis, Churg-Strauss syndrome vasculitis, Henoch-Schönlein purpura and Kawasaki disease;
- 10 - viral infection or replication, e.g. infections due to or replication of viruses including pox virus, herpes virus (e. g., Herpesvirus samiri), cytomegalovirus (CMV), hepatitis viruses or lentiviruses (including HIV);
- asthma, and related respiratory disorders such as allergic rhinitis and COPD;
- osteoporosis; (low bone mineral density);
- 15 - tumor growth;
- organ transplant rejection and/or delayed graft or organ function, e.g. in renal transplant patients;
- a disorder characterised by an elevated TNF- α level;
- psoriasis;
- 20 - skin wounds and other fibrotic disorders including hypertrophic scarring (keloid formation), adhesion formations following general or gynaecological surgery, lung fibrosis, liver fibrosis (including alcoholic liver disease) or kidney fibrosis, whether idiopathic or as a consequence of an underlying disease such as diabetes (diabetic nephropathy);
- 25 - disorders caused by intracellular parasites such as malaria or tuberculosis;

- neuropathic pain (such as post-operative phantom limb pain, post-herpetic neuralgia etc)
- allergies; or
- Alzheimer's disease.

5 According to this invention, further inflammatory disorders include:

- ALS;
- antigen induced recall response
- immune response suppression.

10 These clinical indications fall under the general definition of inflammatory disorders or disorders characterized by elevated TNF α levels.

It should be noted, for the avoidance of doubt, that the primary mechanism of action of the BSCIs, including the compounds claimed herein, is on the immune system. Consequently, the claimed beneficial effects on diseases such as viral infection and/or replication and tumour growth (conditions which, of themselves, are not primarily
15 diseases of the immune system) results from the consequential effects of modulating the immune system on the infection and/or replication patterns of the virus, or on the growth and spread of the tumour. Since BSCIs, including the compounds claimed herein, do not (in general) directly affect viral replication or tumour growth, they would be expected to have no effect at all in an isolated system (for example, in an in
20 vitro infection of a cultured cell line, or in a tumour cell line proliferation assay) where an intact and functioning immune system is absent. Consequently, prior information relating to the effects of any compounds in such isolated systems cannot inform the development of BSCIs, which act on the immune system.

Where legally permissible, the invention also provides a method of treatment,
25 amelioration or prophylaxis of the symptoms of an inflammatory disease (including an adverse inflammatory reaction to any agent) by the administration to a patient of

an anti-inflammatory amount of a compound, composition or medicament as claimed herein.

Administration of a medicament according to the invention can be carried out by topical, oral, parenteral route, by intramuscular injection, etc.

- 5 The administration dose envisaged for a medicament according to the invention is comprised between 0.1 mg and 10 g depending on the formulation and route of administration used.

According to the invention, the compounds of general formula (I) or (I') can be prepared using the processes described hereafter.

10 **Preparation of the compounds of general formula (I) or (I')**

All the compounds of general formula (I) or (I') can be prepared easily according to general methods known to the person skilled in the art.

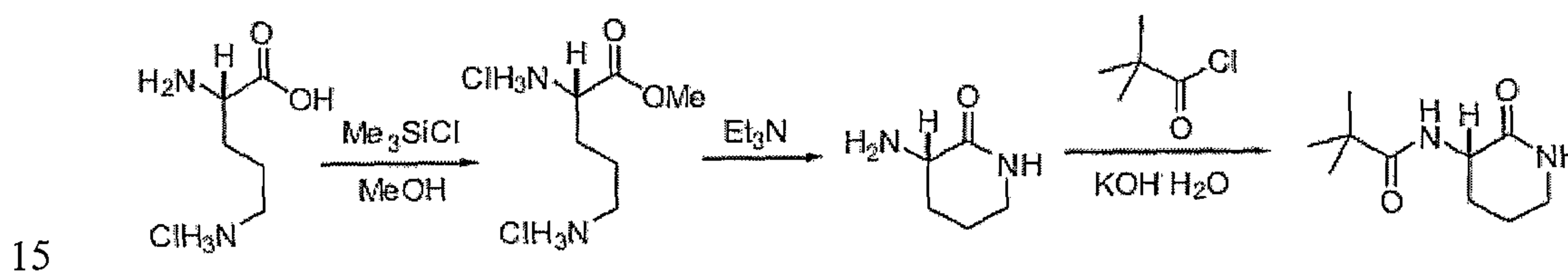
- Compound (I) is a colourless crystalline compound that can be made from ornithine and 2,2- dimethylpropionoyl chloride. For the synthesis of (I') enantiomerically pure
15 (S)-ornithine would be used. A ring closure starting with either ornithine or its methyl ester is possible. The amino acid can be esterified in dry methanol by *in situ* generation of HCl using trimethylsilyl chloride. Alternatively, isolated ester can be ring closed, in either case using triethylamine. The crude product can then be acylated after a solvent exchange.

- 20 The acylaminolactam product (I) has significant water solubility and as a result, the acylation conditions used for related, more hydrophobic, products (see, for example, WO2006/134385) were unsatisfactory. The use of three equivalents of sodium carbonate as base resulted in the formation of significant precipitation of sodium hydrogen carbonate by-product unless a large amount of water was used (>4 mL /
25 mmol ornithine). At these concentrations the extraction of the product into dichloromethane is not efficient. Therefore the three equivalents of sodium carbonate were replaced with 2.5 equivalents of potassium hydroxide (which neutralises the 2.5 equivalents of triethylamine hydrochloride generated in the ring closure step). With

this base significantly less water can be used (less than 1 mL / mmol ornithine) (final pH 8-9). Extraction of the aqueous layer with EtOAc (3 x 2 mL / mmol ornithine) and recrystallisation from EtOAc (0.5 mL / mmol, hot) and 40- 60 petroleum (5 mL / mmol) produced a first crop in 43% yield (4.25 g from 50 mmol ornithine).

- 5 Note that if the pH of the aqueous layer is too low during the work-up then small quantities of triethylamine hydrochloride will be extracted into the EtOAc layer. Attempts at washing this EtOAc solution with water will extract significant quantities of the lactam product (I) along with the amine hydrochloride. This can be avoided by raising the pH of the aqueous layer to 12 (for example by addition of roughly one
- 10 equivalent of KOH with respect to acid chloride) before the EtOAc extraction is attempted, then only triethylamine free base is extracted along with the lactam product (I), which can be removed more easily by evaporation or during recrystallisation.

The following preferred synthetic route is therefore provided:



- (S)-Ornithine monohydrochloride (50 mmol) is suspended in dry methanol (100 ml) and trimethylsilyl chloride (75mmol) added. The reaction is heated at reflux for 24 hours. Triethylamine (150 mmol) is then added and the reaction is heated at reflux for 48 hours. The methanol is then removed under reduced pressure (optionally, toluene
- 20 may be added in the latter stages to facilitate removal), and the residue is dissolved in water (20 mL), with KOH (125 mmol) added.

- The mixture is cooled to 0°C then 2,2-Dimethylpropionyl chloride (50 mmol) is added slowly and the reaction stirred for 18 hours, while warming to ambient temperature. Solid KOH (50 mmol) is then added and once it has dissolved the reaction is extracted
- 25 with EtOAc (3 × 100 mL). The combined organic layers are quickly dried over a combination of K₂CO₃ and Na₂SO₄, and reduced under low pressure. The solid

residue is then recrystallised from EtOAc (25 mL) / 40-60 petroleum ether (200-250 mL) to give the lactam (I') as a crystalline solid (greater than 50% yield).

The identity and purity (>95%) of the product was then confirmed by proton NMR spectroscopy (δ H (400 MHz, CDCl₃) 6.63 (1H, br s, NH), 6.01 (1H, br s, NH), 4.20
5 (1H, dt, *J* 11, 5.5, CHNH), 3.40-3.31 (2H, m, CH₂NH), 2.61 (1H, dq, *J* 13, 4.5, CH₂), 1.97-1.88 (2H, m, CH₂), 1.50 (1H, dddd, *J* 13, 12, 9.5, 7.5, CH₂), 1.22 (9H, s, 3× CH₃).

DEFINITIONS

The term "about" refers to an interval around the considered value. As used in this
10 patent application, "about X" means an interval from X minus 10% of X to X plus 10% of X, and preferably an interval from X minus 5% of X to X plus 5% of X.

The use of a numerical range in this description is intended unambiguously to include within the scope of the invention all individual integers within the range and all the combinations of upper and lower limit numbers within the broadest scope of the given
15 range. Hence, for example, the range of 0.1mg to 10g specified in respect of (*inter alia*) the dose of 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one to be used is intended to include all doses between 0.1mg and 10g and all sub-ranges of each combination of upper and lower numbers, whether exemplified explicitly or not.

As used herein, the term "comprising" is to be read as meaning both comprising and
20 consisting of. Consequently, where the invention relates to a "pharmaceutical composition comprising as active ingredient" a compound, this terminology is intended to cover both compositions in which other active ingredients may be present and also compositions which consist only of one active ingredient as defined.

Unless otherwise defined, all the technical and scientific terms used here have the
25 same meaning as that usually understood by an ordinary specialist in the field to which this invention belongs. Similarly, all the publications, patent applications, all the patents and all other references mentioned here are incorporated by way of reference (where legally permissible).

The following examples are presented in order to illustrate the above procedures and should in no way be considered to limit the scope of the invention.

FIGURES

Figure 1 (panels A to E) shows the time-concentration graphs for the five compounds (I') to (V) tested, following administration of a single 3mg/kg dose in 1% CMC via the oral route to rats. The three lines for each compound represent three replicate animals. The Y-axis represents concentration in units of ng/ml (0-3000); the X-axis represents time in units of minutes (0-480).

Figure 2 shows the major metabolites identified by LC-MS-MS (in full scan mode) on pooled urine collected over 24hrs from rats exposed to a single oral dose of each of the five compounds (I') to (V) tested, administered in 1% CMC at 3mg/kg. Note that a major metabolite of compound (IV) has been identified but its structure was not elucidated by comparison of fragmentation/re-arrangement patterns in the publicly available Metabolite ID databases. Although the concentrations of the individual metabolites were not determined, they are shown in qualitative order of abundance in urine, with the most abundant species on the left of each row.

Figure 3 (panels A-F) shows the current versus time graphs for cells expressing the hERG gene product, when exposed to either vehicle or to the five compounds (I') to (V), each in a separate experiment. In each experiment, replicate cells were exposed to the positive control compound, which completely blocks hERG-transduced current. The Y-axis represents current in units of nA; the X-axis represents time in units of seconds. Panel G shows the hERG tailcurrent (the area under the time versus current graphs in panels A-F) for replicate cells exposed to each compound or to 0.1% DMSO vehicle alone (Veh) or to the positive control compound E-4031 (+ve). The Y-axis of the histogram in panel G represents percentage inhibition of the hERG tail current relative to untreated cells.

Figure 4 shows a typical binding curve for compound (I'). In this experiment, each reaction received 10nM labelled BN83250 (a lactam BSCI which binds to the same receptor as compound (I')), together with various competitors (100µM-1pM). The

total specific binding was the amount displaced by a large excess (100 μ M). Each bar represents the mean of three replicate determinations, with whiskers representing SEM. The Y-axis represents the radioactive counts bound in each experiments in units of counts per minute (cpm). The upper dashed line represents the total binding under the conditions of the experiment, while the lower dashed line represents the non-specific binding; the specific binding is represented by the distance between the dashed lines.

Figure 5 shows the profile of cross-reactivity for each of the five compounds (I') to (V) (panel A: (II); B: (III); C: (V); D: (I') and E: (IV)) against a panel of 75 different receptors that compose the CEREP panel (see text). The compounds were tested at a single concentration (10 μ M), and the inhibition of binding (Y-axis of each histogram) for a known ligand to each of the 75 receptors is reported (-100% therefore represents a 2-fold *increase* in binding of the specific ligand in the presence of the test compound). All reactions were performed in duplicate, and the bars represent the mean (no estimate of the replicate error is shown to simplify the graphs). Only a 50% or greater inhibition (or stimulation) of binding (representing an ED50 below 10 μ M for the interaction of the test compound with the particular receptor) was considered statistically and biologically significant in this screening assay. For the five compounds tested here, only one interaction (that of compound (II) with the NK2 receptor, marked by the arrowhead in panel A) was considered potentially significant, although even this interaction was weak (estimated ED50 5-10 μ M).

Figure 6 (panels A-E) shows representative dose response curves for the inhibition of chemokine-induced leukocyte migration in vitro, for each of the five compounds (I') to (V) in the ChemoTxTM transwell migration assay. In each experiment, THP-1 cells were induced to migrate using a maximally effective dose of the chemokine MCP-1 in the presence or absence of various doses (from 10pM to 1 μ M) of each compound. An appropriate vehicle control was used in each experiment. The percentage inhibition of MCP-1 induced migration (calculated as the number of cells migrated in the presence of MCP-1 minus the number of cells migrated with MCP-1 omitted from the lower chamber) at each concentration of each test compound is shown as the mean of triplicate determinations, with whiskers representing SEM. The ED50 was estimated

by linear interpolation of the presented graphs. The Y-axis of each graph represents the percentage inhibition of MCP-1 induced migration; the X-axis represents the concentration of test compound present in units of nM (0.01-1000).

Figure 7 shows representative dose response curves for the inhibition of LPS induced
5 TNF- α production in vivo in a murine model of sub-lethal endotoxemia. In each experiment, groups of six mice received pre-treatment with the five compounds (panel A: (II); B: (III); C: (V); D: (I') and E: (IV)) at various doses, via either the oral route (circles) or subcutaneous route (triangles). 30-60mins later, animals were challenged with LPS via the intraperitoneal route, and serum was prepared from a
10 terminal bleed 3 hours later. The level of TNF- α in the blood was measured by ELISA, and the degree of inhibition of LPS-induced TNF- α production (calculated as the concentration of TNF- α in mice exposed to LPS minus the concentration of TNF- α in mice which received a sham exposure to endotoxin-free PBS) is shown on the Y-axis of each graph as the mean of six animals, with whiskers representing SEMs. The
15 concentration of TNF- α in mice receiving LPS but no BSCI treatment was typically 5,000 to 6,000 pg/ml on average (compared to <10pg/ml in unchallenged mice). The ED50 was estimated by linear interpolation of the presented graphs. The X-axis of each graph represents the dose of each compound administered to each mouse in the group in units of mg (1E-07 to 1).

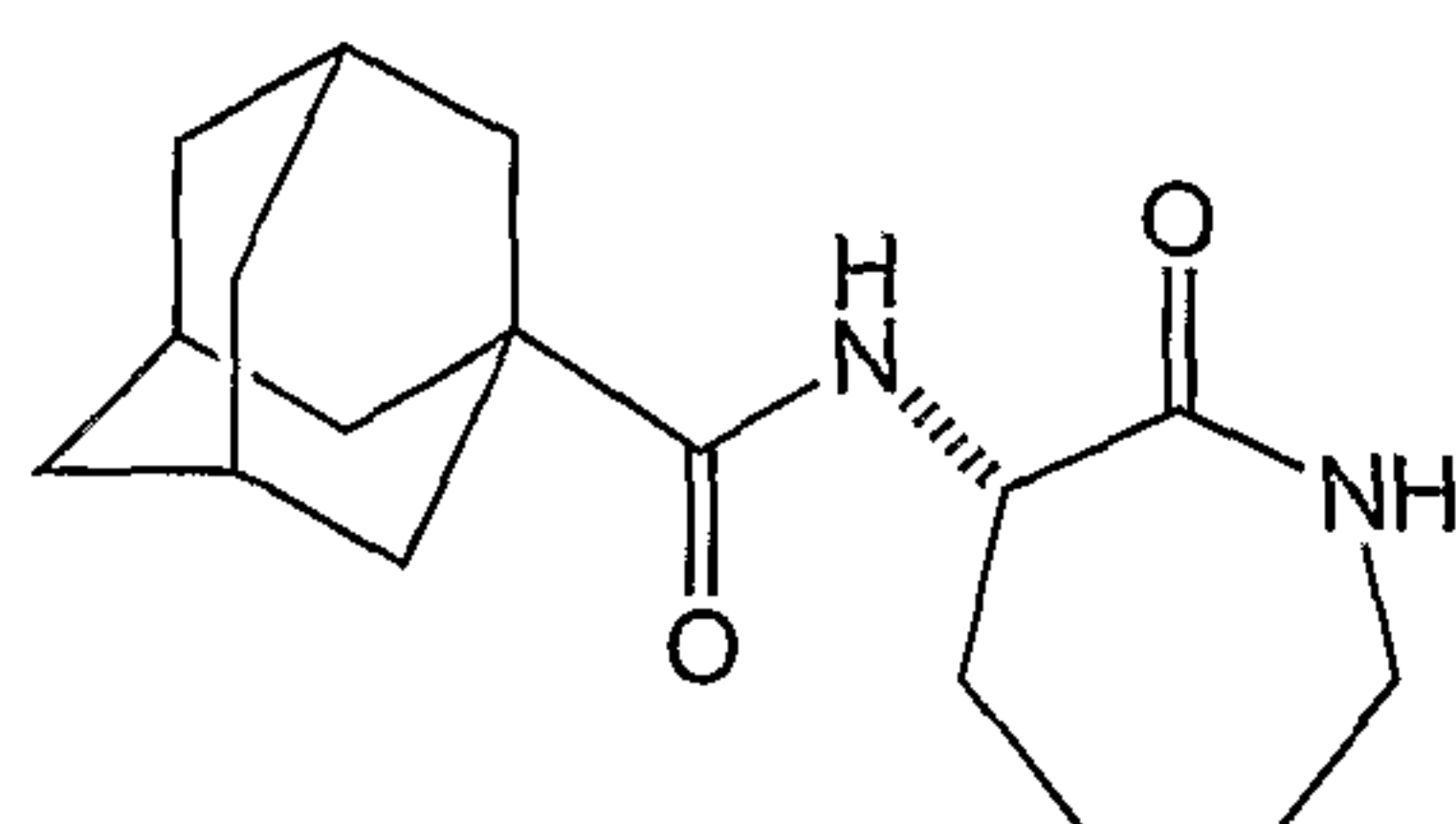
20 Figure 8 shows the effect of compound (I') on lung inflammation in a representative experiment, as assessed by cell counts in BAL fluid in a rodent model of asthma. Bars represent mean cell counts, shown on the Y axis in units of 10^6 cells, for groups of 5 animals with whiskers representing SEM; * $p < 0.01$ versus 'sensitised' using Student's unpaired t-test assuming equal variance, with two tails). The horizontal line
25 represents the average number of leukocytes present in BAL fluid from the lungs of unchallenged rats. All rats in the remaining three groups received the same sensitisation and challenge regimen, but were either treated with vehicle only ('Sensitised'), or with compound (I') at 0.3 mg/kg bodyweight or with monteleukast ('SingulairTM') at 30mg/kg bodyweight, all administered daily by oral gavage.

Figure 9 shows the effect of compound (I') on T-helper cell polarisation in a representative experiment, as assessed by flow cytometric determination of IFN- γ (a Th1 marker cytokine) and IL-4 (a Th2 marker cytokine) production by CD4⁺ splenocytes in a rodent model of asthma. Bars represent mean Th1/Th2 ratios, shown on the Y-axis, for groups of 10 animals, whiskers represent SEM; * $p < 0.05$ versus unchallenged rats; † $p < 0.05$ versus sensitised and challenged rats, in both cases using Student's unpaired t-test assuming equal variance, with two tails). All rats (except the 'BN rats' group, which was not exposed to ovalbumin) received the same sensitisation and challenge regimen, but were either treated with vehicle only ('Sensitised + vehicle'), or with compound (I') at 0.3 mg/kg bodyweight or with monteleukast ('SingulairTM') at 30mg/kg bodyweight, all administered daily by oral gavage.

Figure 10 is a plot of plasma half-life (in minutes) versus oral exposure for a range of different BSCI compounds in rat. In each case, the half-life was estimated using a standard one-compartment model of plasma concentrations at 0, 0.25, 0.5, 1, 2, 4, 6 and 8 hrs after a single iv bolus dose of the compound at 1mg/kg dissolved in 1% CMC, 0.9% DMSO in saline. The oral exposure (AUC_{0-t} in units of min.ng/ml) was calculated following a single oral dose of the compound at 3mg/kg dissolved in 1% CMC, 0.9% DMSO in saline. The R-enantiomer of compound (I) which is the inversion of compound (I') and is identified as "(R)-(I)" is marked with an open circle.

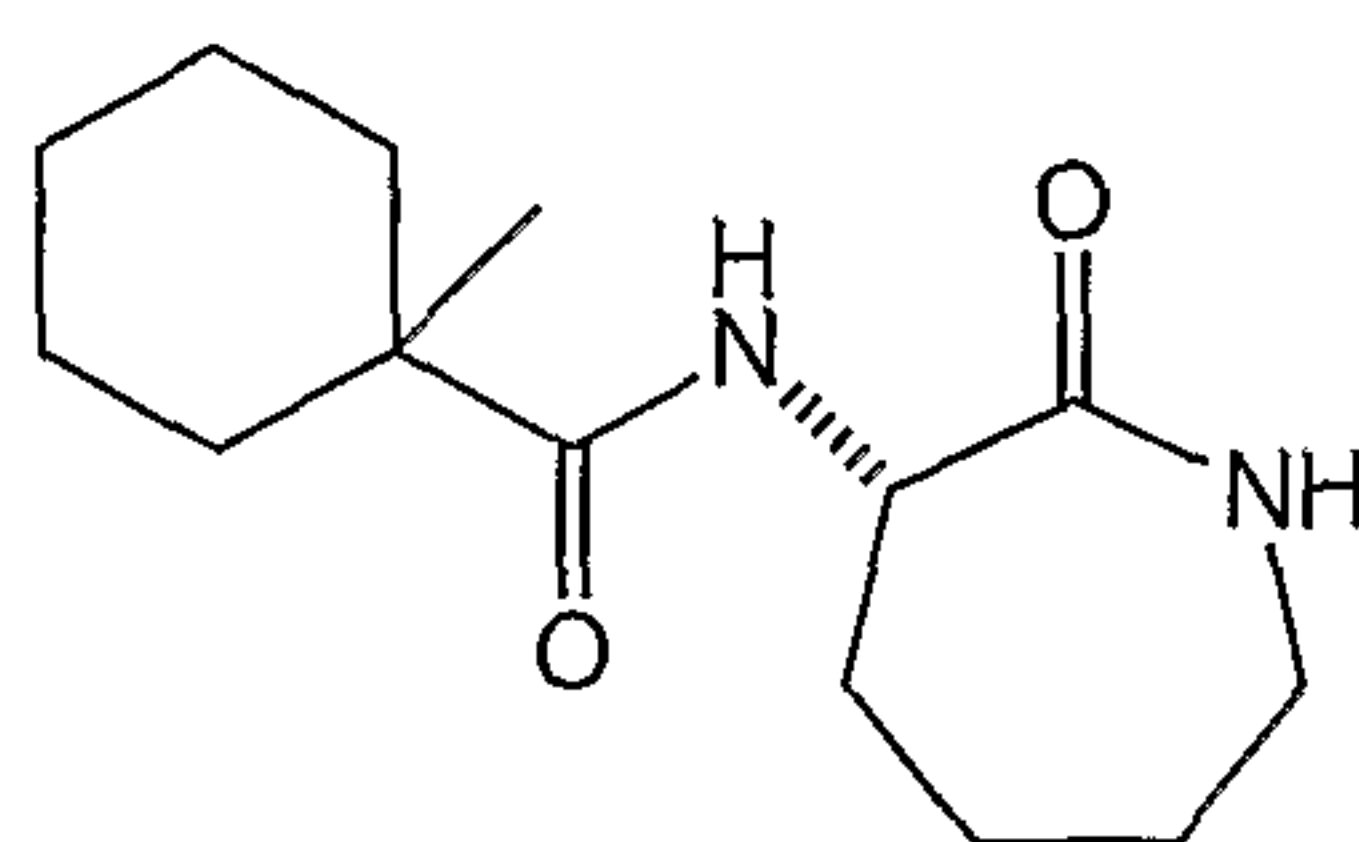
EXAMPLES

In each of the following examples 1 through 6, (S)- 3-(2',2'-dimethylpropanoylamino)-tetrahydropyridin-2-one (compound I') has been compared with a range of other lactam BSCIs which were selected to be representative of the various subclasses. For example, 3-(adamantane-1-carbonylamino)-caprolactam (II) was selected as typical of the subclass of polycycloacyl lactam BSCIs (such as those disclosed previously in WO2006/016152).



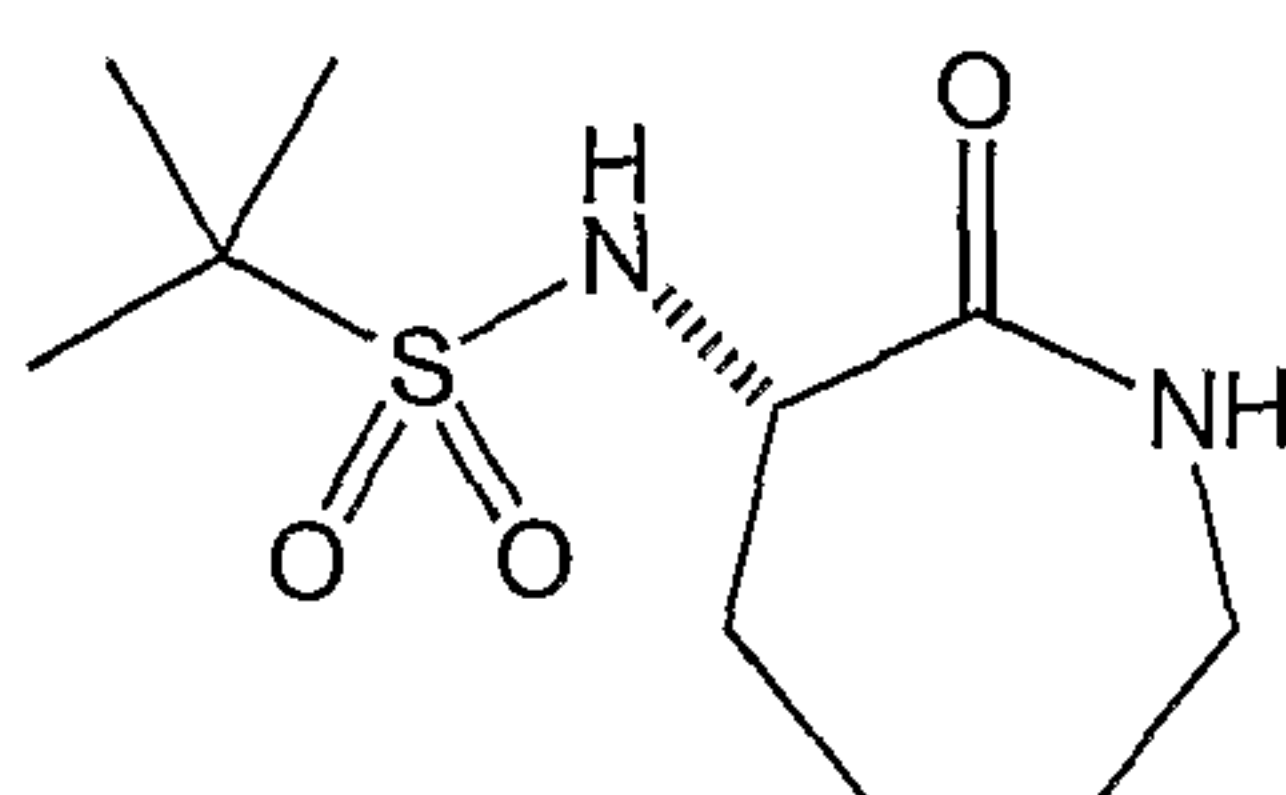
(II)

Similarly, 3-(1'-methylcyclohexylcarbonylamino)-caprolactam (III) was selected as typical of the subclass of monocycloacyl lactam BSCIs (such as those disclosed
5 previously in WO2006/134384).



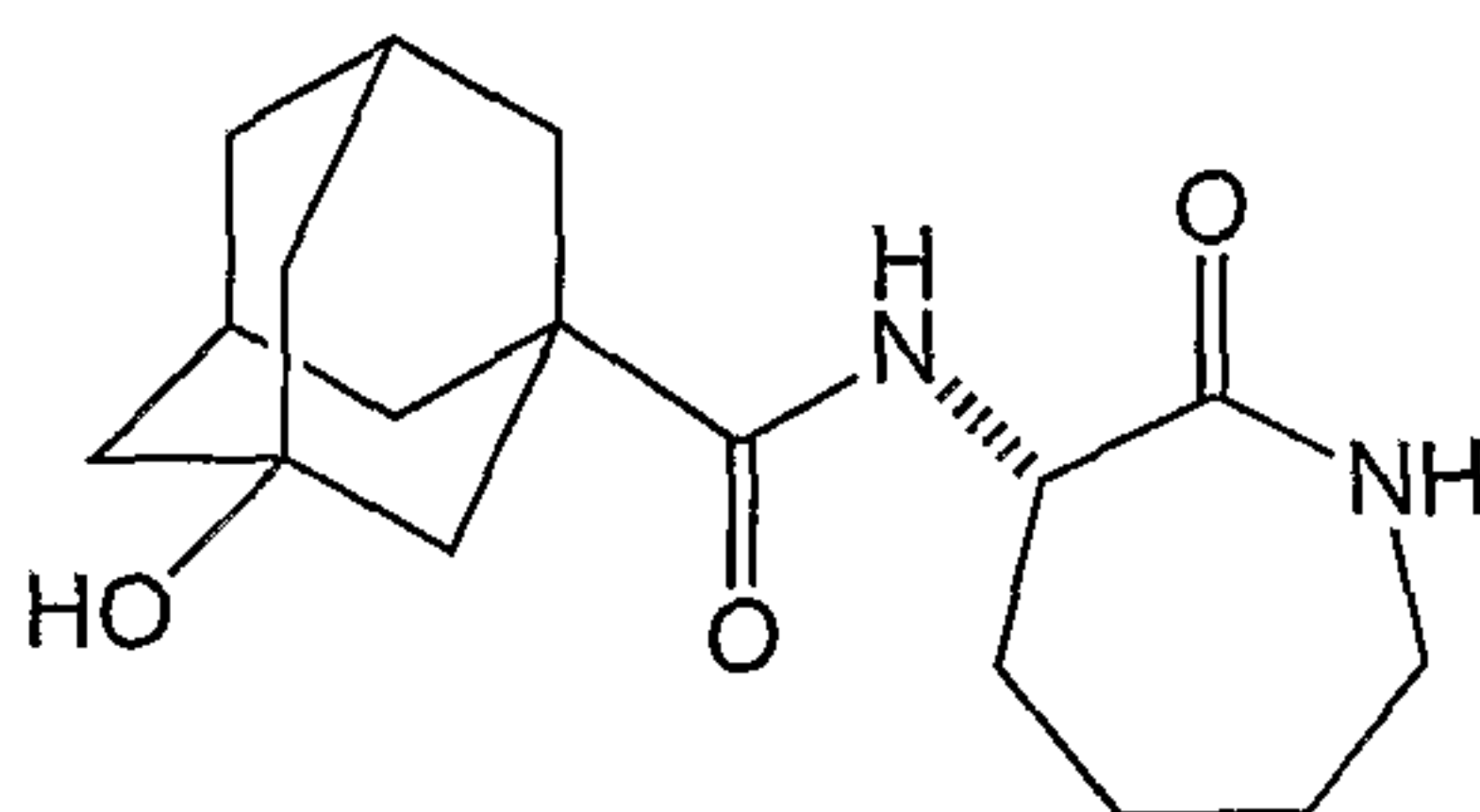
(III)

The compound 3-(1',1'-dimethylethylsulfonylamino)-caprolactam (IV) was selected as typical of the subclass of BSCIs with simple (noncyclic) alkyl side chains (such as
10 those disclosed previously in WO2005/053702), as well as those with a sulfonylamino linker (as opposed to the carbon amide linker in the remaining compounds selected).



(IV)

15 The final compound selected, 3-(3'-hydroxyadamantyl-1-carbonylamino)-caprolactam (V) was typical of BSCIs with a substituted acyl side chain (whether simple linear, branched, mono- or polycyclo in structure).



(V)

It is important to note that all of these BSCI compounds (II) through (V) have been specifically disclosed previously, and all have potent BSCI activity in vitro (ED₅₀ <1nM for the inhibition of MCP-1 induced THP-1 cell migration). All have excellent stability in serum, in vitro, and from a theoretical perspective are all excellent candidates for development as human pharmaceuticals with anti-inflammatory properties in vivo.

All of the above compounds (I', II, III, IV and V) were tested in the (S) configuration at the lactam stereocentre.

Example 1 Pharmacokinetics following a single dose

Compounds were administered as a single dose (either 1mg/kg bodyweight in 5% DMSO via the intravenous route or 3mg/kg in 1% carboxymethylcellulose via the oral route) to three adult rats (using different rats for each compound and each route of administration).

Blood was then sampled at various time points (including just prior to dose administration) out to 24hrs post dose, and the level of the various compounds was assessed using a validated LC-MS/MS assay. Briefly, 3-5µl of the deproteinised sample was applied to a Waters Atlantis (C18 20x2.1mm, 3µm bead size) reverse phase chromatography column equilibrated in 0.1% formic acid in 95 :5 water :acetonitrile. Over 3.5 minutes bound material was gradient eluted reaching 0.1% formic acid in 5 :95 water :acetonitrile, followed by a step gradient back to 0.1% formic acid in 95 :5 water :acetonitrile. The column eluent was then directed to an Applied Biosystems API 4000 / 3200 QTrap MS/MS mass spectrometer, with a

TurboIonspray™ ion source operating in positive ion mode. The interface temperature was set at 650°C, with a 40ms dwell time for each MRM transition, monitoring the following ions :

Analyte	Q1 Mass (amu)	Q3 Mass (amu)
(II)	291.14	135.1
(III)	253.16	97.0
(V)	307.15	151.1
(I')	199.15	57.2
(IV)	249.15	129.3
Internal standard	213.19	57.1

5

The internal standard in each determination was the related compound (S)-3-(2',2'-dimethylpropanoylamino)-caprolactam, which was spiked into the sample prior to deproteinization. The lower limit of quantitation (LLOQ) for this assay was 2.4ng/ml for each compound except (I'), where it was 38.1ng/ml.

- 10 Following LC-MS/MS analysis of each collected sample, the pharmacokinetic disposition of the compound was modelled using Kinetica software, a well known software package for such applications.

Results

- 15 The individual concentration versus time graphs for each rat treated with each compound via the oral route are shown in Figure 1. It is immediately obvious that of these five structurally diverse lactam BSCIs only (IV), (V) and (I') achieve any appreciable oral exposure, and that of these (I') is substantially better than the others.

- 20 The parameters of a simple one-compartment pharmacokinetic model are shown in Table 1. Firstly, this demonstrates the superior exposure achieved with (I') – nearly 20-fold higher than the next best compound, (V). The reason for this superior exposure (which is calculated as the area under the concentration versus time curve, in the units of min.ng/ml) is also evident: the clearance of (I'), which is defined as the theoretical volume of blood which is completely cleared of drug each minute in the units ml/min/kg, is more than 10-times lower than for the other lactam BSCIs.

	F (%)	t _{1/2} (mins)	Clearance (ml/min/kg)	V _{ss} (L/kg)	Exposure (min.ng/ml)
(I')	69	196	2.6	0.7	939,000
(II)	<1	16	84	0.8	477
(III)	5	11	55	0.6	2,900
(IV)	45	19	32	0.6	41,400
(V)	59	24 *	31	1.4	57,100

Table 1. Pharmacokinetic parameters for structurally diverse lactam BSCIs.

Oral bioavailability (F, %), dominant plasma half-life (t_{1/2}, mins), clearance
 5 (ml/min/kg), volume of distribution (V_{ss}, L/kg) and exposure (AUC 0->infinity,
 min.ng/ml) from a simple one-compartment pharmacokinetic model for each
 compound, averaged across three rats. * 24mins is the dominant half-life for (V)
 accounting for the clearance of more than 95% of the injected dose; the minor t_{1/2} β
 was 110mins. In all cases the C_{max} was achieved within 30mins, consistent with
 10 optimal absorption.

The clearance of (II) and (III) approximates to the liver blood flow of the rat,
 strongly suggesting that both these compounds are metabolised almost completely on
 first pass through the liver. Similarly the clearances of both (IV) and (V) exceed
 15 renal blood flow in the rat by some 3-4 fold, again suggesting substantial metabolic
 clearance presumably also liver mediated. In marked contrast, the clearance of (I') at
 2.6 ml/min/kg is less than half of renal blood flow (typically quoted as 7-9
 ml/min/kg), which suggests minimal metabolic clearance. Since (I') is extremely
 water soluble, with a volume of distribution consistent with free equilibration in total

body water (0.7 L/kg), it is likely that the clearance below renal blood flow represents reabsorption with water in the renal distal tubule (rather than, for example, reduced exposure to the kidney due to sequestration into lipophilic compartments).

Consistent with the much lower clearance for (I') compared with the other
5 compounds, (I') has a substantially longer plasma half-life (more than 3 hours, compared with less than half-an-hour for the other four compounds).

Not all of the BSCIs are observed to have oral bioavailability, even though all five of the chosen compounds were known to have oral bioactivity on acute inflammation end-points. This likely reflects the rapid liver-mediated metabolism of (II) and (III),
10 which are absorbed efficiently but converted on first pass through the liver to metabolites which retain some activity as BSCIs (see example 2).

Based on this pharmacokinetic analysis, it will be obvious to those skilled in the art that despite the similar chemical stability and in vitro stability in isolated serum for these five compounds, as well as their similar predicted properties on theoretical
15 grounds, nevertheless (I') is markedly superior to all the others. In particular, the clearance of the compound is much lower, probably reflecting a reduced propensity to liver-mediated metabolism, resulting in a 10-fold longer plasma half-life and almost 20-fold better oral exposure than the next best compound examined.

In a separate experiment, (I') was compared with the second best compound (V) for
20 pharmacokinetic parameters in a different (non-rodent) species, the dog. Single doses (1mg/kg in 5% DMSO intravenously or 3mg/kg in 1% CMC per os) were administered to a single dog for each compound in a simple crossover design with 1 week washout between the two routes of administration.

25

The results are shown in Table 2.

Intravenous PK (1mg/kg)

Compound	Species	Half-life (mins)	Clearance (ml/min/kg)	Vss (L/kg)
(V)	Rat	24	33	0.8
	Dog	66	7.4	0.6
(I')	Rat	196	2.6	0.7
	Dog	236	1.9	0.6

5

Oral PK (3mg/kg)

Compound	Species	Tmax (mins)	Half-life (mins)	Bioavailability (%)	AUC (min.ng/ml)
(V)	Rat*	15	24	59	57100
	Dog	60	67	59	240000
(I')	Rat	15 - 120	226	81	939000
	Dog	60	217	75	1210638

10

Table 2. Comparison of the pharmacokinetic parameters for the best two lactam BSCIs in rat and dog. The pharmacokinetics in dog are broadly similar to rat. In both species, compound (I') having considerably lower clearance, longer plasma half-life and hence greater exposure (AUC in min.ng/ml). In each case the predominant half-life (the faster $t_{1/2\alpha}$) is responsible for clearing more than 95% of the injected dose.

15

These observations indicate that the superior pharmacokinetic properties of (I') are not species specific and are consequently very likely also to be observed in humans.

20

Example 2 Identification of the primary metabolites

Urine was collected from rats exposed to a single oral dose (3mg/kg in 1% CMC) of each compound over a 24 hour period in metabolic cages. The pooled urine sample was then subjected to full scan mass spectral analysis, using the same LC-MS

5 conditions as described in example 1 above. Further MS-MS analysis of the product ions was then performed, and likely fragmentations/re-arrangements assigned from the publicly available Metabolite ID database.

For the major metabolites, the assigned structures were confirmed by synthesis of authentic samples, using methods well known in the art, which were subject to LC-
10 MS-MS analysis under the same conditions as the urine samples.

Note that metabolite ID studies provide only qualitative estimates of the relative amounts of the different metabolites present, and separately validated assays with appropriate internal standards would be required to quantitate each metabolite species.

Results

15 The detected metabolites, in rank order of concentration detected are shown in Figure 2 for the five compounds analysed. It is important to note that the methodology used here is not necessarily exhaustive, and further (particularly minor) metabolites may also be present which were below the detection limits for the methods applied here. As a general rule, it can be assumed that metabolites representing 10% or more of the
20 injected dose will be detected (though not necessarily structurally identified) by the methods used here).

For compounds (II) and (III) the major route of metabolism is cytochrome P450-mediated hydroxylation, consistent with the rapid clearance at a rate approaching liver blood flow (see example 1 above). The major site of hydroxylation in both
25 compounds is on the cycloalkyl tail group, with a second (slower) hydroxylation occurring on the lactam head group. Note that the lactam hydroxylation products appeared in the MS-MS at -2amu (as opposed to +16amu) because of the instability of the 7-hydroxy adducts in the electrospray source.

For compound (II) the dihydroxylated product was present in sufficient quantity to be detected in urine, whereas for compound (III) no dihydroxylation product was detected. In both cases, it is likely that additional minor products were also formed below the level of detection of the method applied (for example, the 3,5 dihydroxy and 3,5,7 trihydroxy adamantyl derivatives of (II), as well as the glucuronidated adducts of both hydroxy-(II) and hydroxy-(III), particularly since the glucuronidated adduct of (V) was detected).

For compound (V) the glucuronidated compound was the major metabolite, although in rat only a minor fraction of the glucuronate is eliminated in urine, the bulk passing into faeces (in marked contrast to humans, where this glucuronidated adduct would be primarily excreted in urine). It is possible that other phase II metabolites (such as the 3'-O-sulphate) were also formed, but only at levels (at least in urine) too low to be detected by the methods used here. As for compounds (II) and (III), a small amount of product hydroxylated on the lactam head group was also detected (again primarily as a -2amu product ion).

For compound (IV) the major metabolite could not be identified, although the loss of parent compound (see Table 3) was clearly consistent with the formation of an unidentified metabolite (less than 10% of the injected dose of (IV) was recovered unchanged. Given that compound (IV) was the only agent containing a sulfonamide linkage it is plausible, but currently unproven, that metabolic cleavage (or other modification) of the linker was occurring. Once again, a small amount of hydroxylation at the lactam head group was also observed.

In marked contrast to all the other compounds, no significant metabolites of (I') were detected in urine, consistent with the appearance of the majority of the injected dose in the urine in unchanged form (see Table 3) and the clearance rates at or below renal blood flow (see example 1 above). This lack of formation of metabolites is a major, and unexpected, advantage of (I') over the other compounds tested here for development as a human pharmaceutical and is, at least in part, responsible for the superior pharmacokinetic properties described in example 1 above.

To provide a quantitative estimate of the degree of metabolism suffered by each of the compounds tested, the amount of unchanged parent compound in urine was measured using the same validated LC-MS assay described in example 1, using (S)-3-(2',2'-dimethylpropanoylamino)-caprolactam as an internal standard. The results are shown in Table 3. In addition, the level of compounds in various target tissues was also determined.

10 *nd = not detected; LLOQ = 2.4 ng/g*

Compound	Heart	Lung	Kidney	Liver	Muscle	Brain	Urine (ng/ml)
(II)	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
(III)	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>
(V)	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	16300
(I')	5.3	21.9	5.1	3.6	3.1	1.6	24567
(IV)	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	<i>nd</i>	1010

Table 3. Distribution of compounds into various tissues 24hrs after a single dose in rat. Only compounds (I'), (V) and (IV) were detectable in urine at 24hrs after a single oral dose (3mg/kg in 1% CMC). Of these, compound (I') underwent significantly less metabolism (more than 60% of the injected dose was recovered in urine). Furthermore, only compound (I') could be detected in any of the other tissues examined 24hrs after a single dose. This likely reflects both the superior distribution and increased exposure associated with (I') compared to the other compounds tested here.

The much lower rate of metabolism of (I') compared to the other compounds demonstrates that, unexpectedly, (I') is markedly superior to the wide range of lactam BSCIs previously disclosed for development as a human pharmaceutical. This
5 reduced metabolism (and hence improved ADME properties) likely accounts for the dramatically superior pharmacokinetic properties shown in example 1 above. Furthermore, since BSCIs are intended for development as anti-inflammatory agents targeting inappropriate leukocyte recruitment into a wide range of tissues, the unexpected finding that (I') is found in all tissues of the body tested 24hrs after a
10 single dose, whereas all the other lactam BSCIs tested were not, unequivocally demonstrates the particular utility of this novel compound.

Example 3. Safety Pharmacology

The five compounds were subjected to a standard AMES test to assess likely
15 genotoxicity. Three His-auxotroph strains of *S. Typhimurium* (TA102, TA98 and TA100) were treated with each of the compounds at 5 concentrations (up to 5mg/ml) in the presence and absence of S9 rat liver microsomal metabolising system. The number of revertant colonies was then determined by plating on trace-His minimal media.

20 The results (Table 4) show that none of the five compounds significantly increase revertant colony formation (with or without metabolic activation) in any of the strains tested.

At 5mg/ml:

* = sparse bacterial background lawn

** = low control value in this experiment

= significant increase in revertant colonies

Compound	TA100	TA100 +S9	TA102	TA102 +S9	TA98	TA98 +S9
(II)	0.59 *	0.73	0.50 *	0.93	0.58 *	0.60 *
(III)	0.79	0.81	0.69	0.88	0.68	0.55
(V)	0.78	0.88	0.93	1.09	0.96	0.91
(I')	0.97	0.95	0.82	0.93	2.00 **	1.23
(IV)	0.89	0.90	0.85	0.88	0.82	0.90
+ve control	6.89	7.18	5.97	2.65	9.68	19.05

5

Table 4. Revertant colony formation in AMES test. None of the compounds tested caused a significant increase in revertant colony formation at any of the concentrations tested (data for the top dose only is shown). Note that compound (II) caused inhibition of bacterial lawn growth at 5mg/ml.

10

In a separate experiment, all five compounds were tested for interaction with the hERG ion channel. Compounds which interact with hERG are at risk of causing QT prolongation and potentially fatal cardiac arrhythmia. Compounds which inhibit hERG tail current by more than 50% at 10 μ M are generally considered high risk for development as human pharmaceuticals.

15

HEK239 cells stably transfected to express hERG were perfused with bath solution containing the compounds at 10 μ M (0.1% DMSO). hERG tail currents from three cells were then recorded by patch-clamp analysis following depolarisation to +20mV

for 5s. The potency of the hERG interaction was then determined in a 4-point dose-response curve for any compounds showing significant modulation at 10 μ M.

The results (Figure 3) show that none of the five compounds significantly interacted with the hERG channel at 10 μ M.

- 5 We conclude that, from a safety pharmacology perspective, all five of the compounds, including (I') are equally suitable for development as human pharmaceuticals. In particular, the considerably superior ADME and pharmacokinetic properties of (I'), illustrated in Examples 1 and 2 above, are not accompanied by correspondingly worse safety pharmacology profiles.

10

Example 4. General pharmacology

- The general pharmacology of the five compounds was assessed, both against the specific target receptor, and against a wide variety of other receptors, many related in structure to the target receptor. Specific binding to the target receptor was assessed
- 15 by competition for the binding of [³H]-BN83250 (BN83250 is (S)-3-(2',2'-dimethyldodecanoylamino)-caprolactam; Fox et al. J Med Chem. 200;48(3):867-74; an agent known to bind to the same target receptor as the lactam BSCIs disclosed here). Binding to non-target receptors was assessed by competition for the binding of various specific radioligands for other receptors which are well known in the art.
- 20 For specific binding the human myelomonocytic cell line was resuspended in binding buffer (20mM HEPES, 150mM NaCl, pH 7.4; 10⁶ cells per reaction) at 4°C in the presence of 10nM [³H]-BN83250 (from 1 μ M stock in 100% ethanol; 30Ci/mmol) and various competitors (1% DMSO maximum vehicle concentration). Reactions were incubated for 2 hours on ice, then filtered through GF/C filters pre-soaked in 0.5%
- 25 polyethyleneimine. Unbound material was washed away with 5x 5ml ice cold washing buffer (20mM HEPES, 150mM NaCl, pH 7.4) under a slow vacuum. These conditions have previously been shown to achieve equilibrium binding, with at least 80% of the binding specific (competable with 10 μ M cold BN83250).

Competition for the specific [^3H]-BN83250 with compounds (I'), (II) and (V) was then determined at various concentrations from 1pM to 10 μM . Compounds (III) and (IV) were not examined in these experiments. A typical competition binding curve for (I') is shown in Figure 4.

- 5 Non-linear modelling was then applied to the competition binding curves for the various compounds, in order to compare their properties as agents binding to the target receptor (defined as the site of specific interaction of BN83250). The parameters of the resulting models are given in Table 5.

Compound	Functional ED50 versus MCP-1 (pM)	Ka at target receptor (pM)	Hill slope
(II)	80	8,200	-0.5
(III)	80	<i>Not tested</i>	<i>Not tested</i>
(V)	120	10,000	-0.5
(I')	50	50	-1.0
(IV)	800	<i>Not tested</i>	<i>Not tested</i>

10 **Table 5. Non-linear modeling of competitive binding curves.**

It is important to note that compound (I'), in marked contrast to lactam BSCIs (II) and (V), showed ideal and predictable binding to the target receptor. In particular, the apparent affinity for binding to the receptor was of a similar magnitude to the functional ED50 value in migration inhibition assays. Similarly, the Hill Slope was approximately -1.0 (the theoretically expected value for a simple, non-cooperative competitive binding model), whereas the other lactam BSCIs showed considerably shallower Hill Slopes. The cause of the departure from ideal binding to the target receptor for compounds (II) and (V) is not known, but this difference further underlines the unexpected superiority of compound (I').

Binding to non-target receptors was assessed using similar protocols, exploiting specific radioligands for each receptor which are well known in the art. Each compound was tested for competition against the specific binding of each ligand only at a single concentration (10 μ M). Where the inhibition of binding was between 20% and 80%, the K_a for the interaction was estimated. Where the inhibition was <20%, the compound was assumed to have no competitive interaction with the receptor. Where the inhibition was >80% the K_a was reported as <1 μ M. Details of the receptors screened, and the radioligands and cell types used in the assays, are available at www.cerep.fr

- 10 The results (Figure 5) demonstrate that all of the lactam BSCI compounds tested are devoid of major cross-reactivities based on this panel of 75 receptors (no interactions with an estimated K_a <1 μ M were noted). Only one weak (but statistically significant) cross-reaction was noted (compound (II) with the NK2 receptor). On this basis, compound (I') was marginally more specific for the target receptor than (II), but all
- 15 the lactam BSCI compounds tested were suitable for development as human pharmaceuticals based on their lack of off-target binding identified in this high throughput screening assay format.

Example 5. Broad-spectrum Chemokine Inhibition activity in vitro

- 20 The biological activity of the compounds of the current invention may be demonstrated using any of a broad range of functional assays of leukocyte migration in vitro, including but not limited to Boyden chamber and related transwell migration assays, under-agarose migration assays and direct visualisation chambers such as the Dunn Chamber.
- 25 For example, to demonstrate the inhibition of leukocyte migration in response to chemokines (but not other chemoattractants) the 96-well format ChemoTx™ micro transwell assay system from Neuroprobe (Gaithersburg, MD, USA) has been used. In principle, this assay consists of two chambers separated by a porous membrane. The chemoattractant is placed in the lower compartment and the cells are placed in the

upper compartment. After incubation for a period at 37°C the cells move towards the chemoattractant, and the number of cells in the lower compartment is proportional to the chemoattractant activity (relative to a series of controls).

This assay can be used with a range of different leukocyte populations. For example,
5 freshly prepared human peripheral blood leukocytes may be used. Alternatively, leukocyte subsets may be prepared, including polymorphonuclear cells or lymphocytes or monocytes using methods well known to those skilled in the art such as density gradient centrifugation or magnetic bead separations. Alternatively, immortal cell lines which have been extensively validated as models of human
10 peripheral blood leukocytes may be used, including, but not limited to THP-1 cells as a model of monocytes or Jurkat cells as model of naïve T cells.

Although a range of conditions for the assay are acceptable to demonstrate the inhibition of chemokine-induced leukocyte migration (see for example the advice provided by Frow et al. Med Res Rev. 2004;24(3):276-98 on the conditions required
15 to interpret in vitro migration assays), a specific example is hereby provided.

Materials

The transwell migration systems are manufactured by Neuroprobe, Gaithersburg, MD, USA. The plates used are ChemoTx™ plates (Neuroprobe 101-8) and 30 µl clear plates (Neuroprobe MP30).

20 Geys' Balanced Salt Solution is purchased from Sigma (Sigma G-9779). Fatty acid-free BSA is purchased from Sigma (Sigma A-8806). MTT, i.e. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is purchased from Sigma (Sigma M-5655). RPMI-1640 without phenol red is purchased from Sigma (Sigma R-8755).

25 The THP-1 cell line (European Cell Culture Collection) were used as the leukocyte cell population.

Test protocol

The following procedure is used for testing the compound of the present invention for the ability to specifically block leukocyte migration induced by chemokines:

First, the cell suspension to be placed in the upper compartment is prepared. The
5 THP-1 cells are pelleted by centrifugation (770 x g; 4 mins) and washed with Geys Balanced Salt Solution with 1mg/ml BSA (GBSS + BSA). This wash is then repeated, and the cells repelleted before being resuspended in a small volume of GBSS + BSA for counting, for example using a standard haemocytometer.

10 The volume of GBSS + BSA is then adjusted depending on the number of cells present so that the cells are at final density of 4.45×10^6 cells per ml of GBSS + BSA. This ensures that there are 100,000 THP-1 cells in each 25 μ l of the solution that will be placed in the upper chamber of the plate.

To test a single compound for its ability to inhibit chemokine induced migration, it is necessary to prepare two lots of cells. The suspension of THP-1 cells at 4.45×10^6
15 cells/ml is divided into two pots. To one pot the inhibitor under test is added at an appropriate final concentration, in an appropriate vehicle (for example at 1 μ M in not more than 1% DMSO). To the second pot an equal volume of GBSS + BSA plus vehicle as appropriate (e.g. not more than 1% DMSO) is added to act as a control.

Next, the chemoattractant solution to be placed in the lower compartment is prepared.
20 For example, MCP-1 is diluted in GBSS + BSA to give a final concentration of 25 ng/ml. This is divided into two pots, as for the cell suspension. To one pot, the test compound is added to the same final concentration as was added to the cell suspension, while to the other pot an equal volume of GBSS + BSA plus vehicle as appropriate (e.g. not more than 1% DMSO) is added. Alternatively, other
25 chemokines may be used (SDF-1 α at 7.5ng/ml; RANTES at 50ng/ml; IL-8 at 10ng/ml using neutrophils as the target cell population). In each case it is important to determine (in a separate experiment) the concentration of each chemokine which causes maximal stimulation of migration of the chosen target leukocyte population. This maximal concentration must then be used in experiments to test the inhibitory

activity of the compounds of the invention. Because chemokines typically induce leukocyte migration with a bell-shaped dose-response curve, the use of a sub- or supra-maximal chemokine concentration can lead to artefactual results (for example, a compound which is a chemokine inhibitor can yield a paradoxical *stimulation* of leukocyte migration if a supra-maximal concentration of chemoattractant is incorrectly selected for the experimentation. Further illustrations of this important factor in the design of in vitro leukocyte migration experiments has are provided by Frow and colleagues (*Med Res Rev.* 2004;24(3):276-98). In addition, non-chemokine chemoattractants may also be used to demonstrate the chemokine-selectivity of the biological activity of the compounds of the invention (for example C5a at 25ng/ml using neutrophils as the target cell population or TGF- β 1 at 10ng/ml using THP-1 cells as the target population).

Note that the volume of liquid that needs to be added with the addition of the test compound needs to be taken into account, when establishing the final concentration of MCP-1 in the solution for the lower compartment and the final concentration of cells in the upper compartment.

Once the chemoattractant solutions for the lower wells and cell solutions for the upper chambers have been prepared, the migration chamber should be assembled. Place 29 μ l of the appropriate chemoattractant solution into the lower well of the chamber. Assays should be performed with at least triplicate determinations of each condition. Once all the lower chambers have been filled, apply the porous membrane to the chamber in accordance with the manufacturer's instructions. Finally, apply 25 μ l of the appropriate cell solution to each upper chamber. A plastic lid is placed over the entire apparatus to prevent evaporation.

The assembled chamber is incubated at 37 °C, 5% CO₂, for 2 hours. A suspension of cells in GBSS + BSA is also incubated under identical conditions in a tube: these cells will be used to construct a standard curve for determining the number of cells that have migrated to the lower chamber under each condition.

At the end of the incubation, the liquid cell suspension is gently removed from the upper chamber, and 20 μ l of ice-cold 20mM EDTA in PBS is added to the upper

chamber, and the apparatus is incubated at 4°C for 15 mins. This procedure causes any cells adhering to the underside of the membrane to fall into the lower chamber.

After this incubation the filter is carefully flushed with GBSS + BSA to wash off the EDTA, and then the filter is removed.

- 5 The number of cells migrated into the lower chamber under each condition can then be determined by a number of methods, including direct counting, labelling with fluorescent or radioactive markers or through the use of a vital dye. Typically, we utilise the vital dye MTT. 3 µl of stock MTT solution are added to each well, and then the plate is incubated at 37 °C for 1-2 hours during which time dehydrogenase
10 enzymes within the cells convert the soluble MTT to an insoluble blue formazan product that can be quantified spectrophotometrically.

In parallel, an 8-point standard curve is set up. Starting with the number of cells added to each upper chamber (100,000) and going down in 2-fold serial dilutions in GBSS + BSA, the cells are added to a plate in 25 µl, with 3 µl of MTT stock solution
15 added. The standard curve plate is incubated along side the migration plate.

At the end of this incubation, the liquid is carefully removed from the lower chambers, taking care not to disturb the precipitated formazan product. After allowing to air dry briefly, 20µl of DMSO is added to each lower chamber to solubilise the blue dye, and absorbance at 595nm is determined using a 96-well plate
20 reader. The absorbance of each well is then interpolated to the standard curve to estimate the number of cells in each lower chamber.

The chemoattractant stimulated migration is determined by subtracting the average number of cells that reached the lower compartment in wells where no chemoattractant was added from the average number of cells that reached the lower
25 compartment where the chemoattractant was present.

The impact of the test substance is calculated by comparing the chemoattractant-induced migration which occurred in the presence or absence of various concentrations of the test substance. Typically, the inhibition of migration is expressed as a percentage of the total chemoattractant-induced migration which was

blocked by the presence of the compound. For most compounds, a dose-response graph is constructed by determining the inhibition of chemoattractant-induced migration which occurs at a range of different compound concentrations (typically ranging from 1nM to 1μM or higher in the case of poorly active compounds). The inhibitory activity of each compound is then expressed as the concentration of compound required to reduce the chemoattractant-induced migration by 50% (the ED₅₀ concentration). Typically, MCP-1 induced migration of THP-1 cells has been used as the standardised test system for the comparison of the biological activity of a wide range of compounds (see for example Reckless & Grainger *Biochem J.* 1999 Jun 15;340 (Pt 3):803-11; Reckless et al. *Immunology.* 2001 Jun;103(2):244-54; Fox et al. *J Med Chem.* 2002 Jan 17;45(2):360-70; Fox et al. *J Med Chem.* 2005 Feb 10;48(3):867-74; the International applications *supra*). Compounds which inhibit leukocyte migration induced by more than one chemokine, but not by non-chemokine chemoattractants (such as TGF-β or C5a) are defined as Broad-spectrum Chemokine Inhibitors (BSCIs; see for example Grainger & Reckless *Biochem Pharmacol.* 2003 Apr 1;65(7):1027-34; Grainger et al. *Mini Rev Med Chem.* 2005 Sep;5(9):825-32).

Results

A typical dose response curve for compound (I') inhibiting MCP-1 induced migration of THP-1 cells is shown in Figure 6, together with comparable dose response curves for other selected lactam BSCIs known to have particularly high (that is, <1nM) potency. The potency of compound (I') versus various chemokines and non-chemokine chemoattractants, expressed as ED50 values is shown in Table 6, and is compared with other lactam BSCIs described previously.

It is clear from this data that compound (I') can be classified as a BSCI (since it powerfully and potently inhibits leukocyte migration induced by a range of chemokines, but has no effect on leukocyte migration induced by a non-chemokine chemoattractant, in this case the C5a anaphylatoxin). Furthermore, it is evident that compound (I') is at least as potent and powerful as a BSCI in vitro as the selected lactam BSCIs which have previously been disclosed (for example, compound (II) in WO2006/016152 or compound (IV) in WO2005/053702). All of the lactam BSCIs examined here are considerably more potent than any of the non-lactam BSCIs which

have been disclosed to date (including imides, such as NR58,4, yohimbamides, lysergamides and peptide 3 and related structures such as NR58-3.14.3). Indeed, compound (I') is more potent as a BSCI in vitro (at least against MCP-1 induced migration) than any other compound disclosed or described previously. Although this potency as a BSCI is quantitatively superior to BSCIs in the prior art (albeit to a small degree), it is not this property that primarily marks out compound (I) as unexpectedly superior to the prior art BSCIs. Instead, this demonstrates that the unexpected, and substantially superior, ADME and pharmacokinetic properties of compound (I) compared to a wide range of previously disclosed BSCIs, has been achieved with no loss of power or potency as a BSCI in vitro.

Compound	MCP-1	SDF-1 α	RANTES	IL-8 neutrophils	C5a neutrophils
(II)	80	100	100	600	>1,000,000
(III)	80	<i>Not tested</i>	<i>Not tested</i>	<i>Not tested</i>	<i>Not tested</i>
(V)	120	200	250	500	>1,000,000
(I')	50	50	80	600	>1,000,000
(IV)	800	<i>Not tested</i>	<i>Not tested</i>	<i>Not tested</i>	<i>Not tested</i>

Table 6. Effect of selected lactam BSCIs on leukocyte migration in vitro. In each case the dose of compound (in pM) required to inhibit leukocyte migration in response to a maximal dose of the stated chemoattractant by 50% (the ED50) is shown. Unless stated otherwise, the data is reported for the THP-1 cell lines. For C5a induced migration none of the compounds tested inhibited neutrophil migration to any degree even at the highest concentration tested (1 μ M).

Example 6. Anti-inflammatory activity in vivo

We have used the sub-lethal LPS-induced endotoxemia assay to demonstrate the generalised anti-inflammatory properties in vivo of previously disclosed BSCIs (see, for example, Fox et al. *J Med Chem.* 2002;45(2):360-70; Fox et al. *J Med Chem.* 2005;48(3):867-74). In this assay, mice are given a non-specific pro-inflammatory challenge using bacterial endotoxin (LPS), and the extent of the systemic inflammatory response (measured by serum levels of the central pro-inflammatory cytokine TNF- α , which is essentially absent from the blood under normal conditions, but is rapidly elevated in response to a wide range of inflammatory stimuli). We have selected this model even though it is not, itself, a particularly close model of any human inflammatory disease condition, but because TNF- α is known to be important in very many diseases (including rheumatoid arthritis, autoimmune disorders, Crohn's Disease, atherosclerosis, asthma and many more). Consequently, agents which suppress TNF- α production are already used clinically (e.g. Enbrel™ and other anti-TNF- α antibody products) to treat a wide range of such diseases. Demonstration of TNF- α suppressive activity in this model is therefore highly predictive of a clinically useful anti-inflammatory effect in a wide range of diseases.

Mice (in groups of 6) were pretreated with various doses of each compound, either by the subcutaneous route 30 mins prior to LPS challenge, or by the oral route (via gavage) 60 mins prior to LPS. The mice were then challenged with an intraperitoneal injection of 750 μ g of bacterial LPS and sacrificed 3 hours later. Serum was prepared from a terminal bleed by cardiac puncture, and the concentration of TNF- α is determined by ELISA (R&D Systems). In each experiment, a group of 6 mice receive no LPS to act as a negative control, and a second group receive only LPS (with no candidate inhibitor). The level of TNF- α in serum from these animals, which received LPS without drug pre-treatment, is arbitrarily set to 100% (and is typically of the order of 6,000 pg/ml, compared with levels of <10pg/ml among the negative control group). We have previously shown that the synthetic corticosteroid dexamethasone (itself a well known anti-inflammatory medicament active in a wide range of inflammatory diseases) inhibits LPS-induced TNF- α production by at least

90% in this model, while thalidomide (another published inhibitor of TNF- α production, acting at the level of cellular TNF- α production rather than as a leukocyte recruitment inhibitor like the BSCIs described here) inhibits LPS-induced TNF- α production by about 60%.

- 5 The effect of compound (I') at various doses, as well as other selected lactam BSCIs, is shown in Figure 7. As expected, the compound powerfully inhibits LPS-induced TNF- α production whether the compound is delivered via the subcutaneous route (circles) or oral route (triangles). At doses above 1 μ g/mouse, LPS-induced TNF- α levels were generally suppressed by more than 90%, comparable to the effects of the
10 corticosteroid dexamethasone.

- The other lactam BSCIs tested also inhibited LPS-induced TNF- α in a dose-dependent manner (Figure 7), although the potency in vivo of (I') was greater than any of the other compounds tested (and, indeed, greater than the potency of other lactam BSCIs previously disclosed elsewhere which have been tested in this assay).
15 This quantitative (albeit small) increase in potency is not the primary reason that we hereby claim compound (I) as unexpectedly superior to the prior art BSCIs. Instead, this demonstrates that the unexpected, and substantially superior, ADME and pharmacokinetic properties of compound (I) compared to a wide range of previously disclosed BSCIs, has been achieved with no loss of power or potency as an anti-
20 inflammatory agent in vivo. In addition, these findings clearly demonstrate the utility of (I) as an anti-inflammatory agent in vivo, in a model of inflammation which indicates utility in a wide variety of inflammatory diseases where increased TNF- α production is a component of the pathogenic mechanism.

- It is important to note that the hyperacute inflammation observed in this model is
25 particularly insensitive to the ADME and pharmacokinetic properties of the anti-inflammatory agents tested. Since the LPS stimulation is administered only 30 minutes after the drug, even agents with very short plasma residence times (such as compounds (II) and (III)) remain present in plasma at sufficient concentrations to elicit a powerful anti-inflammatory effect. While such a test, therefore, does not

emphasise the superiority of the claimed compounds over the prior art, it nevertheless demonstrates the utility of the compound.

The utility of the claimed compound is further demonstrated by studies in an animal model of the human disease asthma (the hyperacute inflammatory response observed in response to LPS exposure may not be typical of any particular human disease, although it is clearly a useful model system of acute inflammation in general). In these studies, rodents (typically rats) are exposed to ovalbumin in accordance with the following experimental design:

Adult Brown Norway rats (200-300g body weight; n=10 per group) were sensitised by a single intraperitoneal injection of 0.1mg Ovalbumin on day 0. Each rat then received an intratracheal challenge with a solution of 1% ovalbumin (w/v) on day 8, and with 2% ovalbumin (w/v) on days 15, 18 and 21. The animals were then sacrificed 3 hours after the final challenge on day 21. Note that ovalbumin (Sigma; purest available grade) was made endotoxin-free by passage over EndoTrap™ Red columns (purchased from Cambrex; used in accordance with the manufacturer's instructions), and the endotoxin level was confirmed as <5 EU/mg protein using the LAL assay (QCL-1000™; Cambrex; performed in accordance with the manufacturer's instructions; 1mg of standard endotoxin contains ~900,000 EU/mg). This ensures that the lung inflammation response results from the allergic response to the ovalbumin protein, rather than from unintended LPS stimulation which occurs even with the highest purity grade commercial ovalbumin preparations, and therefore ensures the model more closely represents the underlying molecular pathology of human asthma.

One group of mice (acting as a baseline control) received no ovalbumin challenges, but were otherwise treated identically. A second group (positive control) received the challenges but no drug treatment. A third group were treated identically, but received daily dosage with compound (I') at a dose of 0.3mg/kg via oral gavage from day 8 until day 21, with dosage being given 1hr prior to any subsequent challenge with ovalbumin made on the same day. Compound (I') was administered as a sterile solution in endotoxin-free phosphate buffered saline. A fourth group received monteleukast (the active component of the commercially available asthma medication

Singulair™) at 30mg/kg via the oral route, in an identical treatment schedule to compound (I').

On sacrifice, total lung leukocyte recruitment was assessed by performing a bronchoalveolar lavage (BAL) using 4 lots of 3ml sterile phosphate-buffered saline introduced through a tracheal cannula. For each animal, the BAL washes were combined, and the total cell population counted (using a haemocytometer).

Additionally, the types of leukocyte present were estimated using a flow cytometer in accordance with procedures well known in the art.

The spleen was also removed from each mouse and placed in RPMI +10% FCS + antibiotics. The spleen was then pressed through fine-mesh (100µm) nylon screens in a sterile sieve cup placed in a sterile petri dish to produce single-cell suspensions. The resulting cell suspension was then centrifuged (328g; 5 mins) and washed in RPMI +10% FCS + antibiotics, before being resuspended in fresh media and counted using a haemocytometer.

4x10⁶ total splenocytes (excluding RBCs) in total were cultured (37°C; 5% CO₂) in RPMI+10%FCS + antibiotics overnight in presence of 2U/ml (10ng/ml) murine IL-2 in 4 wells of a 96 well plate (100µl volume per well/1x10⁶cells/well) from each mouse. Approximately 24hrs later, the 4 wells were split into two groups of 2 wells: one group were left untreated, while the second group were stimulated with 500ng/ml Ionomycin and 50ng/ml PMA for 4 hours at 37°C. During the last two hours of this incubation 10µg/ml Brefeldin A (stock 1mg/ml in EtOH) was added to one well from each set. Brefeldin A blocks protein transport to golgi and therefore allows accumulation of proteins in ER.

The wells without Brefeldin A were incubated for a further 48 hours at 37°C. At the end of the incubation, the cell suspensions were centrifuged (328g; 5 mins) and the supernatant was subjected to ELISA assays (R&D Systems; performed in accordance with the manufacturer's instructions) for murine IL-4 (a marker of Th2 cells) and murine interferon-γ (IFN-γ; a marker of Th1 cells).

The wells with Brefeldin A were stained for intracellular IL-4 and IFN-g immediately at the end of the four hour incubation as follows: cells were stained with anti-CD4-FITC antibody (eBioscience Rat IgG2b, Cat. Code. 11-0041) for 30 mins on ice, then washed in Dulbecco's PBS and fixed with 2% paraformaldehyde (final concentration) in Dulbecco's PBS for 20 mins. After fixation cells were made permeable with Dulbecco's PBS/1%BSA/0.5% saponin (Sigma S7900) for 10 mins at room temperature. The cells from each well were then split into three separate FACS tubes and incubated with:

- IFN-g-PE (eBioscience Rat IgG1, Cat. Code. 12-7311-82, 100 μ g) OR
- IL-4-PE (eBioscience Rat IgG1, Cat. Code. 12-7041-82, 100 μ g) OR
- Isotype controls (a mixture of Rat IgG2b-FITC, eBioscience Cat. Code 11-4031 and Rat IgG1-PE, eBioscience Cat. Code 12-4301)

for 30 mins at room temperature. Cells were then washed (twice with PBS/BSA/saponin and then with PBS/BSA without saponin to allow membrane closure) and resuspended in Dulbecco's PBS ready for flow cytometry analysis.

Cells with specific staining for CD4 on the FITC channel (identifying them as T-helper cells) were analysed for the presence of specific staining for either IL-4 or IFN-g on the PE channel. The ratio of CD4+ cells staining positive for IFN-g to CD4+ cells staining positive for IL-4 was then reported as the Th1/Th2 ratio. Untreated Brown Norway rats have a Th1/Th2 ratio of approximately 2.7 in the spleen (that is, approximately 2.7 times more CD4+ cells in the spleen are synthesising INF-g as IL-4). Following sensitisation and repeated challenge with ovalbumin, the ratio had fallen to less than 1.5 demonstrating the marked Th2 polarisation which accompanies asthmatic changes in both rodents and humans (a lower Th1/Th2 ratio indicates relative Th2 polarisation, while an increasing Th1/Th2 ratio indicates a relative Th1 polarisation).

Daily dosing with both compound (I') and the positive-control comparator compound monteleukast significantly reduced the number of leukocytes in the BAL washes (70% reduction with compound (I'); $p < 0.01$ Student's unpaired t-test; Figure 8).

This unequivocally demonstrates that the compound of the invention has a useful anti-inflammatory effect in a model of human asthma, resulting from its ability to block leukocyte migration in response to chemokines. The magnitude of such an effect is at least comparable with that of commercially available medicaments intended for the treatment of human asthma (such as SingularTM), while the excellent pharmacokinetic and biodistribution parameters of compound (I') are illustrated by the considerably increased potency compared to monteleukast (a dose of 30mg/kg of monteleukast is required to generate a similar reduction in BAL leukocyte counts compared to a dose of only 0.3mg/kg of compound (I')).

Daily dosing with compound (I'), but not with the positive-control comparator compound monteleukast, significantly reversed the Th2 polarisation (Figure 9), which is considered a major driver of asthma pathogenesis both in the ovalbumin-induced lung inflammation model used here, and in human asthma. Treatment with compound (I') even at low doses such as 0.3mg/kg via the oral route completely abolishes the Th2 polarisation caused by chronic exposure to allergens such as ovalbumin, such that the Th1/Th2 balance in animals treated with compound (I') is essentially indistinguishable from unchallenged Brown Norway rats.

It is interesting to note that other chronic inflammatory diseases, such as atherosclerosis, are associated with a Th1 (as opposed to a Th2) polarisation. In both types of diseases, the imbalance in the T-helper cell cytokine profile has been described as a major pathogenic cause of the chronic inflammatory component of the disease. In models of a disease associated with a Th1 polarisation (such as in atherosclerosis) we have previously observed a marked shift towards Th2 on treatment with BSCIs, such as compound (I') claimed here. Mice with a homozygous deletion of the gene encoding apoE (apoE^{-/-} mice) develop several vascular lipid lesions, even on a normal chow diet, and have a Th1/Th2 ratio of approximately 8 (compared to 3.2 in the background C57Bl6 wild-type strain). However, following treatment with BSCIs for a 3 month period (from 12 weeks of age to 24 weeks of age, the period during which most of the lipid lesion development occurs) normalises the Th1/Th2 ratio (and even causes a Th2 polarisation at very high doses in this model). Taken together with the data in the ovalbumin-induced lung inflammation model of

asthma, we have demonstrated that BSCIs are able to normalise or rebalance the T-helper cell cytokine production profile irrespective of whether the underlying pathogenic defect is a Th2 polarisation (as in asthma) or a Th1 polarisation (as in atherosclerosis). We believe that BSCIs are currently the only agents described which have this “rebalancing” effect on the T-helper cell population. These mechanistic insights further underpin (together with efficacy data in numerous animal models of different diseases with an inflammatory component) our claim that BSCIs, and in particular compound (I) claimed here (as a result of its unexpectedly superior pharmacokinetic and biodistribution properties) are useful as medicaments to treat an unusually broad range of conditions with an inflammatory component.

Example 7. Comparison of compound (I') with structurally related analogs

Having identified compound (I) as having surprisingly superior pharmaceutical properties, particularly with respect to its pharmacokinetics, compared to other acylaminolactam BSCIs, we next compared it directly with the most structurally similar analogs that have previously been disclosed.

The original panel of compounds compared in examples 1 through 6 had been assembled to be as structurally diverse as possible, within the boundaries of the previously disclosed acylaminolactam BSCIs. Compound (I) ensured that 6-membered lactam ‘headgroups’ were included in the panel, although there is no particular reason to assume they are generally superior (or indeed inferior) to the compounds with 7-membered lactam rings (see WO2006/134385 for an extensive comparison of acylaminolactam analogs of different ring sizes in terms of potency as BSCIs in vitro). Similarly, the panel included a single example of a sulfonamide linkage (IV) as distinct from the amide linker in the remaining molecules.

With respect to the ‘tailgroups’, the panel included two simple alkyl groups (both pivoyl) and three cyclic alkyl groups (a methylcyclohexyl group, and two adamantanes, one of which was substituted). All five compounds shared the well-established 2'2-disubstitution with tetrahedral bond angles at the key carbon (at the 2

position relative to the carbonyl of the amide carbonyl, with 4 carbon atoms bonded to this key carbon in a tetrahedral arrangement). This 2',2'-disubstitution feature at the key carbon atom has already been disclosed (WO2006/016152) as conferring additional potency on BSCIs compared to linear alkyl 'tailgroups', or other structures
5 lacking four carbon atoms bonded to this key carbon in an essentially tetrahedral arrangement.

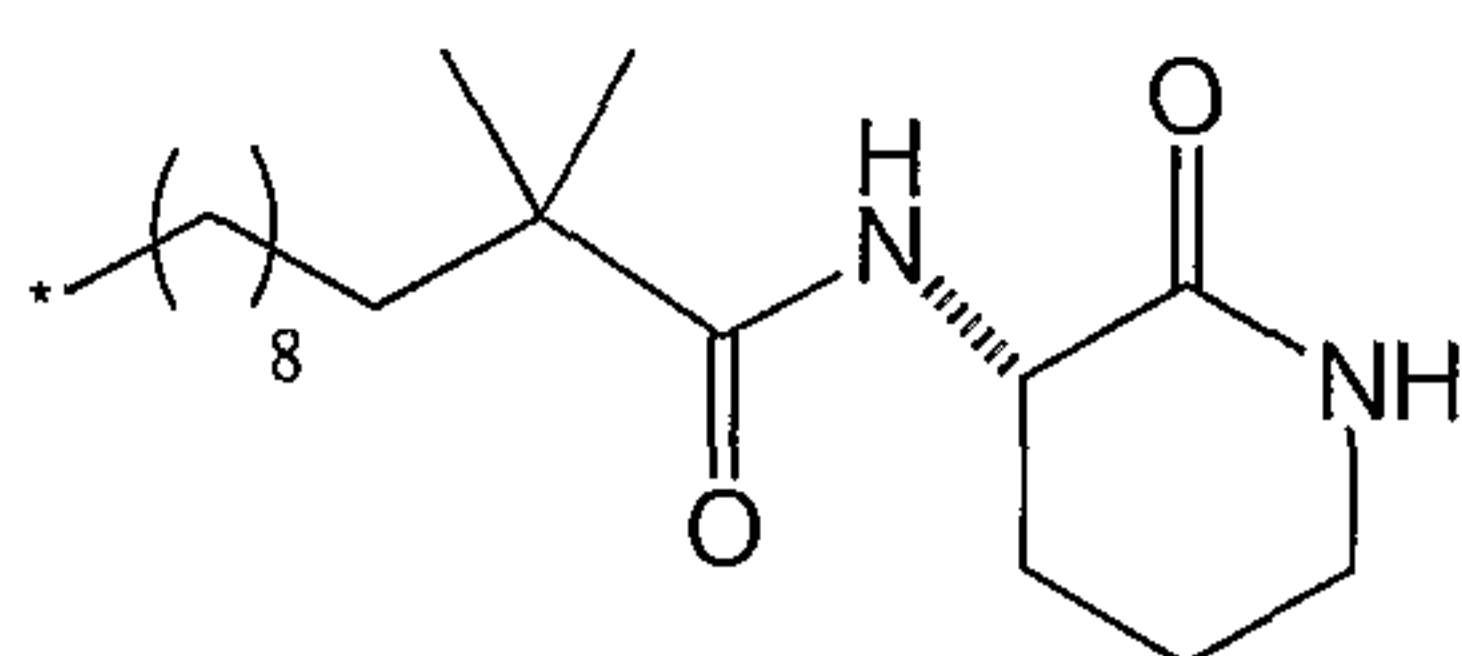
As a result, the 'tailgroups' in the original panel were selected with the requirement of having this 2',2'-disubstitution feature, to ensure adequate in vitro potency, but also with the requirement of likely yielding acceptable physical properties in the resulting
10 molecules. For example, it was expected that the adamantane, cyclohexyl and short chain 2',2'-branched alkyl groups would result in compounds with crystalline (or semi-crystalline) solid forms, and with acceptable water solubility. In contrast, other possible tailgroups, such as 2',2'-dimethyldodecanoyl (which otherwise conforms to the desirable arrangement of carbon atoms at the key 2-position), were excluded from
15 the panel on the basis of their waxy, amorphous physical forms as solids and their extreme insolubility in physiological buffers. Both of these factors reduce the desirability of a compound for pharmaceutical development even before the ADME and toxicological properties have been determined because they make the compound difficult to handle, for example during formulation or tableting, and this increases the
20 likelihood of unfavourable ADME properties such as accumulation in fat stores during chronic dosing or lack of oral bioavailability.

Thus the five compounds examined in examples 1 through 6 had a roughly equal chance of being suitable for pharmaceutical development. Prior to determining their ADME properties it was not possible, to predict which would be superior.

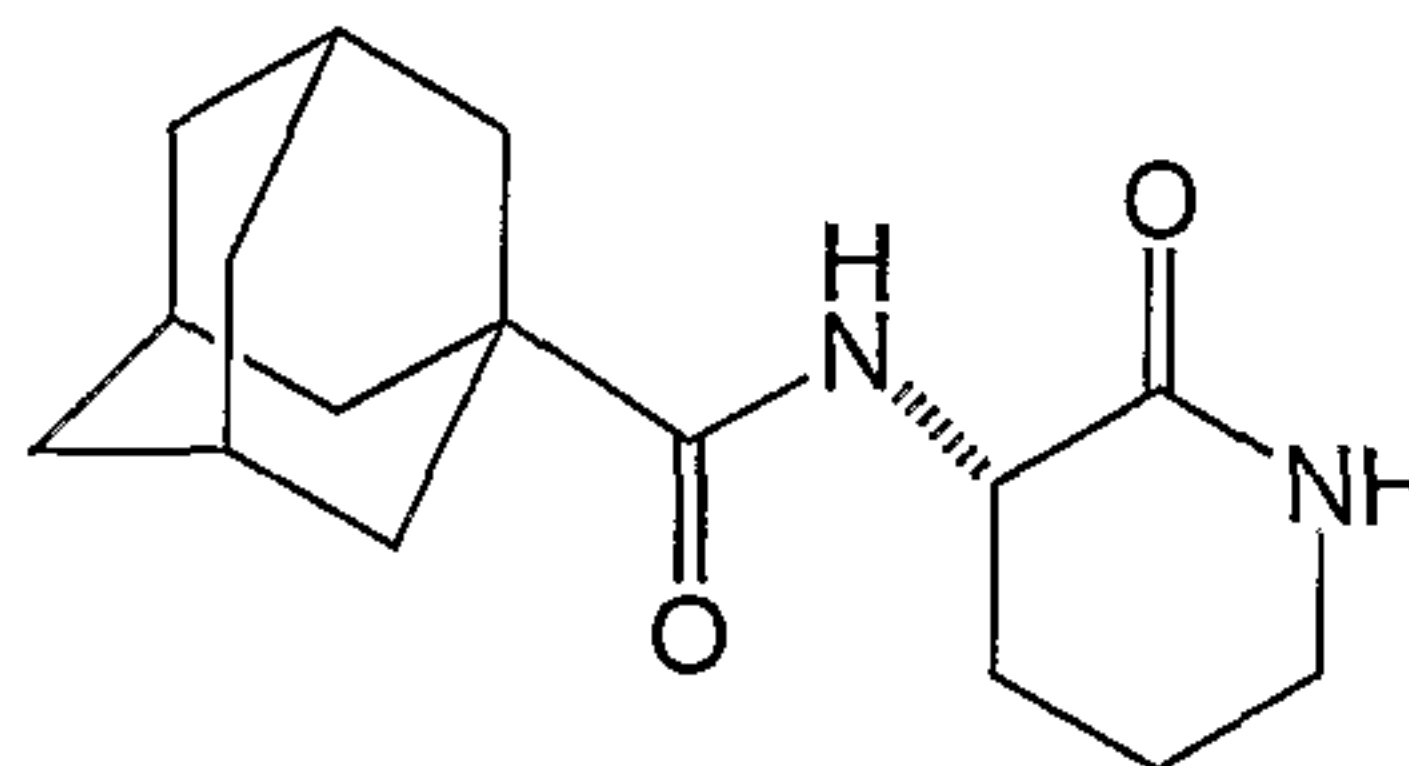
25 Once the testing described in examples 1 through 6 was complete, however, it became clear than compound (I) was substantially and unexpectedly superior to the other compounds. Since compound (I) was the only compound in the panel with a 6-membered ring, it was possible that the superior properties were due to the size of the lactam ring. To determine whether this was true, we reviewed all the previously
30 disclosed examples of acylaminolactam compounds with BSCI activity, and selected the two compounds with greatest structural similarity to (I). Of all the previously

known compounds with BSCI activity, these were the two compounds with the most similar structure to compound (I).

The selected compounds were 3-(2',2'-dimethyldodecanoylamino)-tetrahydropyridin-2-one (example 3 from WO2006/134385; here designated **VI**) and 3-(adamanatane-1-carbonylamino)-tetrahydropyridin-2-one (example 1 from WO2006/134385; here designated **VII**):



(VI)



(VII)

These compounds share in common with (I) the 6-membered lactam ring, the amide linker and the presence of 2',2'-disubstitution, with 4 carbon atoms bonded to the key carbon at the 2-position relative to the amide carbonyl group, in an essentially tetrahedral arrangement.

Compounds (VI) and (VII) were subject to pharmacokinetic analysis in rat using exactly the same protocols previous applied for compounds (I) through (V) in example 1. Note that the (S) enantiomer of both (VI) and (VII) was used for these experiments, since this is expected to be the more active enantiomer in common with all other acylaminolactam BSCIs described to date.

Results

Unfortunately, (VI) was a waxy solid with a tendency to retain solvent. It was difficult to obtain a preparation of (VI) which was solvent-free, but this was eventually achieved after considerable drying over many weeks. However, this sample was completely insoluble in the 5% DMSO in saline, used as the vehicle for intravenous dosing of the previous compounds. As a result, it was necessary to use a wholly different vehicle in order to prepare a solution suitable for intravenous dosing.

Compound (VI) was dissolved in 50% PEG400 / 20% solutol / 30% saline for intravenous dosing. All compounds, including (VI), were suspended (or dissolved) in 1% carboxymethylcellulose in saline for oral dosing. Although comparing oral bioavailability between experiments using different vehicles is not generally recommended (because the vehicle can have a considerable impact on gut absorption of compounds), it is nevertheless very unlikely that the selection of vehicle significantly affects the observed half-life following intravenous injection of (VI) compared with other compounds, such as (I).

The parameters of a simple one-compartment pharmacokinetic model are shown in Table 7. Data for compound (I') from Table 1 above is shown for comparison. It is evident that (VI) and (VII) are both markedly inferior to (I'), with clearance rates more than 10-fold higher, and oral exposure which is 25-50 fold less than was achieved with (I')

	F (%)	t _{1/2} (mins)	Clearance (ml/min/kg)	V _{ss} (L/kg)	Exposure (min.ng/ml)
(VI)	16	46	40.3	1.95	11,000
(VII)	35	20	31.1	0.7	32,500
(I')	69	196	2.6	0.7	939,000

Table 7. Pharmacokinetic parameters for selected 6-ring acylaminolactam BSCIs. Oral bioavailability (F, %), dominant plasma half-life (t_{1/2}, mins) following intravenous dosing, clearance (ml/min/kg), volume of distribution (V_{ss}, L/kg) and oral exposure (AUC 0-t, min.ng/ml) from a simple one-compartment pharmacokinetic model for each compound, averaged across three rats. For (VII), C_{max} was achieved within 15mins, consistent with optimal absorption as for (I'). The hydrophobic (VI) was absorbed more slowly, however, with T_{max} between 60 and 120mins.

Indeed, it is evident from comparison of Table 7 with Table 1 that compounds (VI) and (VII) are not even as good as (IV) and (V) in terms of exposure. Despite sharing the 6-lactam ring with (I), compounds (VI) and (VII) have markedly inferior ADME properties, and are no better than a wide range of other acylaminolactam BSCI compounds which have previously been disclosed.

Consistent with the much higher clearance for (VI) and (VII) compared with (I'), these compounds have substantially shorter plasma half-lives than (I') (less than 1 hour, compared with more than 3 hours).

Based on this pharmacokinetic analysis, it will be obvious to those skilled in the art that despite the similar chemical stability, as well as their similar predicted properties on theoretical grounds and their similarity in structure, nevertheless (I') is markedly superior to both (VI) and (VII), with 25-50 fold better oral exposure than the either of these 6-ring lactam analogs. We conclude that the unexpectedly excellent ADME properties of (I') cannot be attributed to the 6-ring lactam (since other 6-ring lactams, such as (VI) and (VII) do not share its outstanding ADME characteristics), nor to the amide linker (since other amide linker compounds, including (II), (III) and (V) do not share its outstanding ADME characteristics), nor to the pivoyl tail group (since compound (IV) shares this group and does not have the outstanding ADME characteristics of (I')). Instead, the surprising and markedly superior ADME characteristics of (I') depend on the particular combination of structural features, which could not be predicted.

Example 8. Attempt to rationally design an acylaminolactam BSCI with ADME properties equivalent to (I')

In the process of identifying the superior ADME properties of compound (I'), we have analysed the pharmacokinetics of a variety of different acylaminolactams with sub-nanomolar potency as BSCIs in vitro. As a result, we would expect to have gained some insight into the factors which modulate ADME properties for molecules within this structural space. We therefore attempted to use this experience to select a

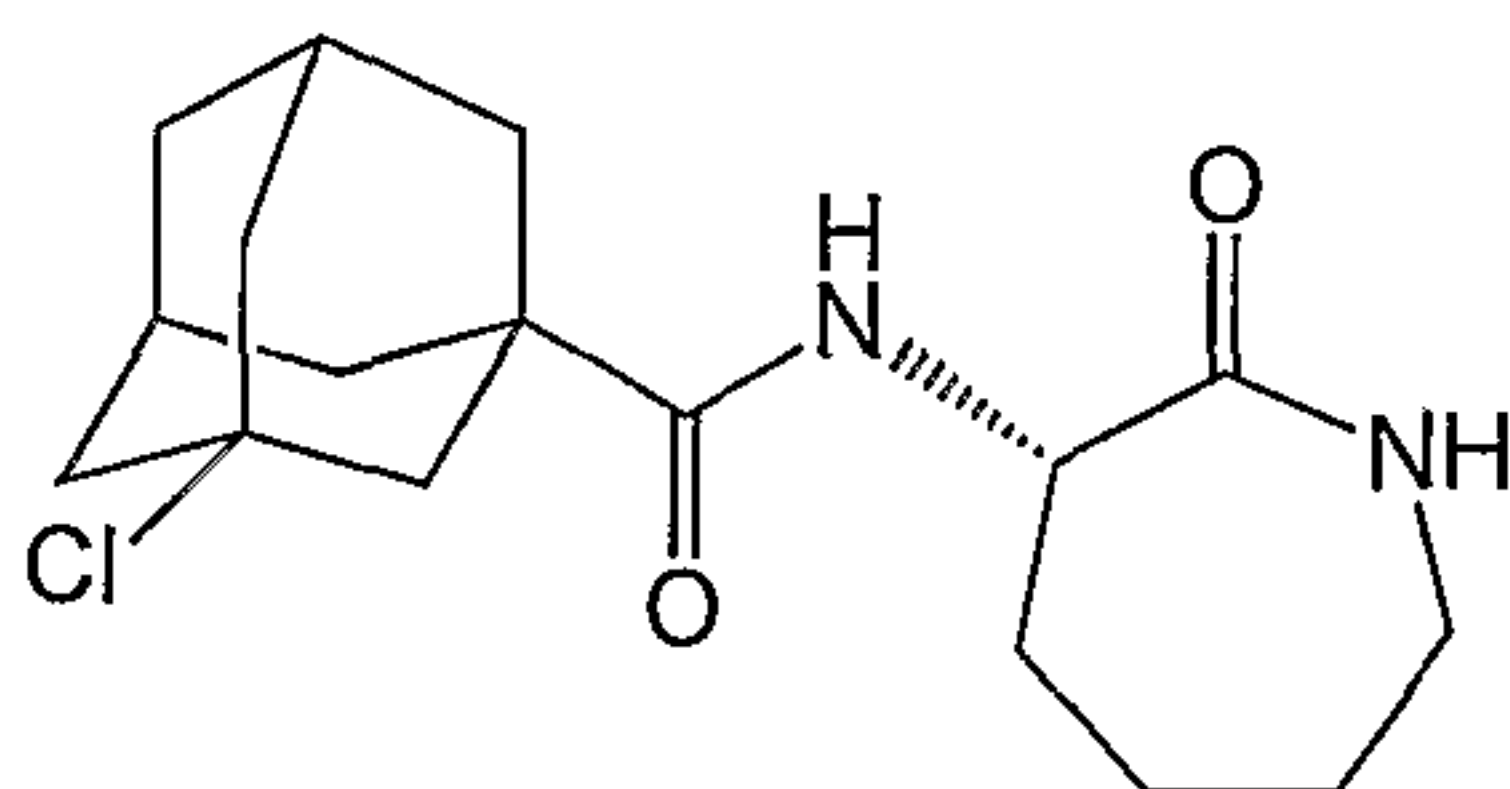
second acylaminolactam BSCI compound from amongst the structural classes previously disclosed with ADME properties as similar as possible to those of (I').

The relatively rapid clearance of (IV) compared with (I') (see Table 1 above) suggested that the amide linker may be correlated with superior ADME properties.

5 We therefore limited the selection to compounds with amide linkers.

Compound (II) was very well absorbed, but was rapidly converted to (V) in vivo, presumably through the action of liver cytochrome P450 isoenzyme(s). Compound (V) itself was not apparently further oxidised (probably as a result of the electron withdrawing substituent on the adamantane ring) but was nevertheless cleared
10 relatively rapidly. The absence of detectable primary metabolites in the urine strongly suggests that the rapid clearance was mediated by the formation of a phase II conjugate, such as the glucuronide, which in rat is excreted in the faeces. Phase II conjugates are readily formed at hydroxyl groups, such as the 3-hydroxyadamantane in (V). As a result, we proposed that a derivative of (II) with a stable electron-
15 withdrawing substituent (to prevent hydroxylation of the adamantane ring by cytochrome P450 or other liver hydroxylases or oxidases) which was unable to form phase II conjugates, such as a halo substituent, may have the optimal ADME properties we were seeking.

As a result, compound (VIII), below, was synthesised and tested. We selected the
20 chloro-substituted analog (over the fluoro-substituted analog, for example) because this was a known compound (see example 8 in WO2006/016152) for which a synthetic route was readily available. Furthermore, introduction of aliphatic C-F bonds is challenging synthetically, and can increase the cost of goods for the active pharmaceutical ingredient by an order of magnitude or more.



(VIII)

As previously (see Example 7), we elected to prepare and evaluate the (S)-enantiomer of this compound, since in general the (S)-enantiomers of acylaminolactams compounds are known to have greater BSCI activity in vitro.

5 An obvious concern related to this molecule was the stability of the C-Cl bond. We therefore examined the stability of (VIII) in vitro, both in physiological saline and in a rat liver microsome preparation. Encouragingly, less than 2% of (VIII) had undergone hydrolysis (determined by LC/MS, with dual ion detection of the hydrolysis product (V) and the parent compound (VIII)) even after 24 hours at 37°C. Similarly, incubation of (VIII) at 1 µM with a rat microsome preparation for 1 hour at 10 37°C resulted in less than 10% degradation compared to a “no NADPH” blank run. As a result, we conclude that compound (VIII) has similar in vitro stability and intrinsic clearance to (I’).

Thus, based on theoretical grounds using the experience gained from the discovery of (I’), as well as general skill in the art of predicting ADME properties, as well as using 15 conventional in vitro screening assays, such as in vitro stability and intrinsic clearance in liver microsome preparations, compound (VIII) would be expected to have ADME properties comparable with (I’).

We therefore subjected (VIII) to pharmacokinetic analysis in rat using exactly the same protocols previously applied for compounds (I’) through (VII) in examples 1 and 20 7.

Results

The parameters of a simple one-compartment pharmacokinetic model are shown in Table 8. Data for compound (I’) from Table 1 above is shown for comparison. It is evident that compound (VIII) is markedly inferior to (I’), with clearance rates more 25 than 20-fold higher, and no detectable oral bioavailability whatsoever.

	F (%)	t _{1/2} (mins)	Clearance (ml/min/kg)	V _{ss} (L/kg)	Exposure (min.ng/ml)
(VIII)	<1%	15	62.8	0.7	<100
(I')	69	196	2.6	0.7	939,000

Table 8. Pharmacokinetic parameters for selected lactam BSCIs. Oral

bioavailability (F, %), dominant plasma half-life (t_{1/2}, mins) following intravenous dosing, clearance (ml/min/kg), volume of distribution (V_{ss}, L/kg) and oral exposure (AUC 0-t, min.ng/ml) from a simple one-compartment pharmacokinetic model for each compound, averaged across three rats. For (VIII), the level of analyte was below the limit of quantitation (~2ng/ml) at all time-points following oral dosing.

In conclusion, compound (VIII), which we had selected on the basis of theoretical considerations and in vitro screening tests, to have optimal ADME properties was nevertheless among the least suitable acylaminolactam compounds, at least amongst those whose PK has been determined, for development as a pharmaceutical agent. The compound exhibited rapid clearance, resulting in a short plasma half-life and additionally showed no oral bioavailability whatsoever.

The extent of the superiority of compound (I') is illustrated in Figure 10. Here, we have plotted two PK parameters (plasma half-life and oral exposure) for every BSCI compound for which the pharmacokinetics have been analysed in rat. Compound (I') lies far away from the cluster, representing the generally poor ADME properties of compounds in this structural space (and not untypical of very many entirely unrelated classes of drug candidates), indicating that compound (I') is unexpectedly, and unusually, superior.

Example 9. Comparison of the (S)- and (R)-enantiomers of (I)

Compound (I') has been shown to have unexpected, and markedly superior, ADME properties (see example 1). This compound has an assymetrical carbon atom (at the ring junction with the amide linker) and consequently exists in two stereoisomers: the
5 (S)-enantiomer designated (I') and the opposite (R)-enantiomer, designated (R)-(I).

As with all other acylaminolactam BSCIs described to date, the (S)-enantiomer of (I) is more potent as a BSCI in vitro than the corresponding (R)-enantiomer. It is for this reason that (I') has been studied in most experiments, rather than the isolated (R)-enantiomer or a mixture of the enantiomers. However, the ADME properties are
10 unlikely to be related to the primary pharmacological activity. Indeed, for many compounds the ADME properties of enantiomeric pairs are very similar, since (in many cases) the physical properties of the compound are particularly dominant in determining the in vivo disposition, and the physical properties of enantiomeric pairs are identical.

15 As a result, we tested whether the (R)-enantiomer shares the particularly excellent ADME properties of (I'). Since the utility of any particular compound depends on both the potency of the primary pharmacological effect(s) and on the disposition of the molecule in vivo (since to be efficacious, the drug molecule must be present at the intended target site(s) at a sufficient concentration to exert its pharmacological
20 effects), then a compound that has lower potency but excellent ADME properties may be more efficacious (or more potent in vivo) than a comparator compound of greater potency but inferior ADME properties.

The isolated (R)-enantiomer of (I) was therefore synthesised (exactly as described herein for the isolated (S)-enantiomer, but replacing the (S)-ornithine starting material
25 with commercially available (R)-ornithine of high stereochemical purity), and the PK properties of this agent, in rat, was determined using the identical methodology described above (see Examples 1, 7 and 8).

Results

The parameters of a simple one-compartment pharmacokinetic model are shown in Table 9. Data for compound (**I'**) from Table 1 above is shown for comparison. It is evident that (R)-(**I**) shares, to a large extent, the unexpectedly superior ADME properties of (**I'**). As with (**I'**), (R)-(**I**) is also essentially quantitatively orally bioavailable, and achieves an exposure following a single oral dose at 3mg/kg more than 5-times higher than the next best compound tested to date (see compound (**V**) in example 1; Table 1).

	F (%)	t _{1/2} (mins)	Clearance (ml/min/kg)	V _{ss} (L/kg)	Exposure (min.ng/ml)
(R)-(I)	84	81	8.6	0.9	295,000
(I')	69	196	2.6	0.7	939,000

Table 9. Pharmacokinetic parameters for the isolated (R)- and (S)-enantiomers of (I**).** Oral bioavailability (F, %), dominant plasma half-life (t_{1/2}, mins) following intravenous dosing, clearance (ml/min/kg), volume of distribution (V_{ss}, L/kg) and oral exposure (AUC 0-t, min.ng/ml) from a simple one-compartment pharmacokinetic model for each compound, averaged across three rats. In both cases, peak plasma concentration was typically observed by 30mins, consistent with good absorption.

Although (R)-(**I**) clearly shares, to a significant extent, the unusually beneficial ADME properties of (**I'**), there are nevertheless some statistically significant differences. Most marked is the increased plasma clearance (~3-fold higher for (R)-(**I**) compared with (**I'**)), resulting in a 2-fold shorter plasma half-life following intravenous dosing as well as a 3-fold lower exposure following the single oral dose.

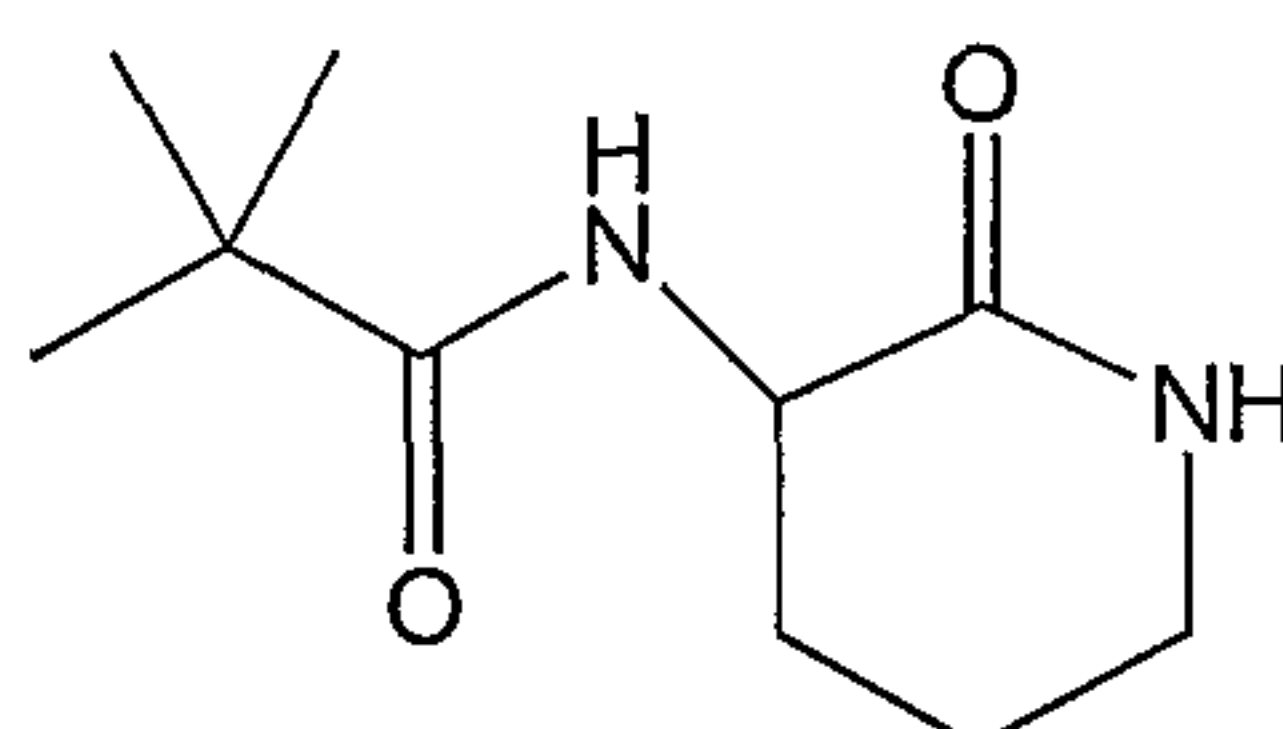
The clearance of (R)-(I) approximates the glomerular filtration rate in rat (typically quoted at 9 ml/min/kg), whereas the clearance of (I') is significantly lower. Once possible interpretation of these findings is that (I') is actively reabsorbed, perhaps in the distal tubule of the nephron, and this reabsorption process is selective for the (S)-
5 enantiomer over the (R)-enantiomer. If, as seems plausible, the unexpected and highly superior ADME properties of (I') are due, in part, to reuptake in the nephron via a transporter capable of distinguishing the (S)-enantiomer from the (R)-enantiomer then it underlines the unpredictability of ADME properties, and further illustrates the non-obviousness of the invention described herein.

10 Nevertheless, (R)-(I) is clearly also a superior compound according to the present invention (as illustrated by the open circle in Figure 10, which illustrates that it is closer in ADME properties to (I') than to the general class of acylaminolactams. In conclusion, therefore, both (R)-(I) and (I'), and hence more generally compound (I) is shown to have unexpectedly, and markedly, superior ADME properties compared to
15 other acylaminolactam BSCIs which have been disclosed. Moreover, compound (I') is slightly, but significantly, superior to compound (I) generally, and is therefore preferred.

Claims

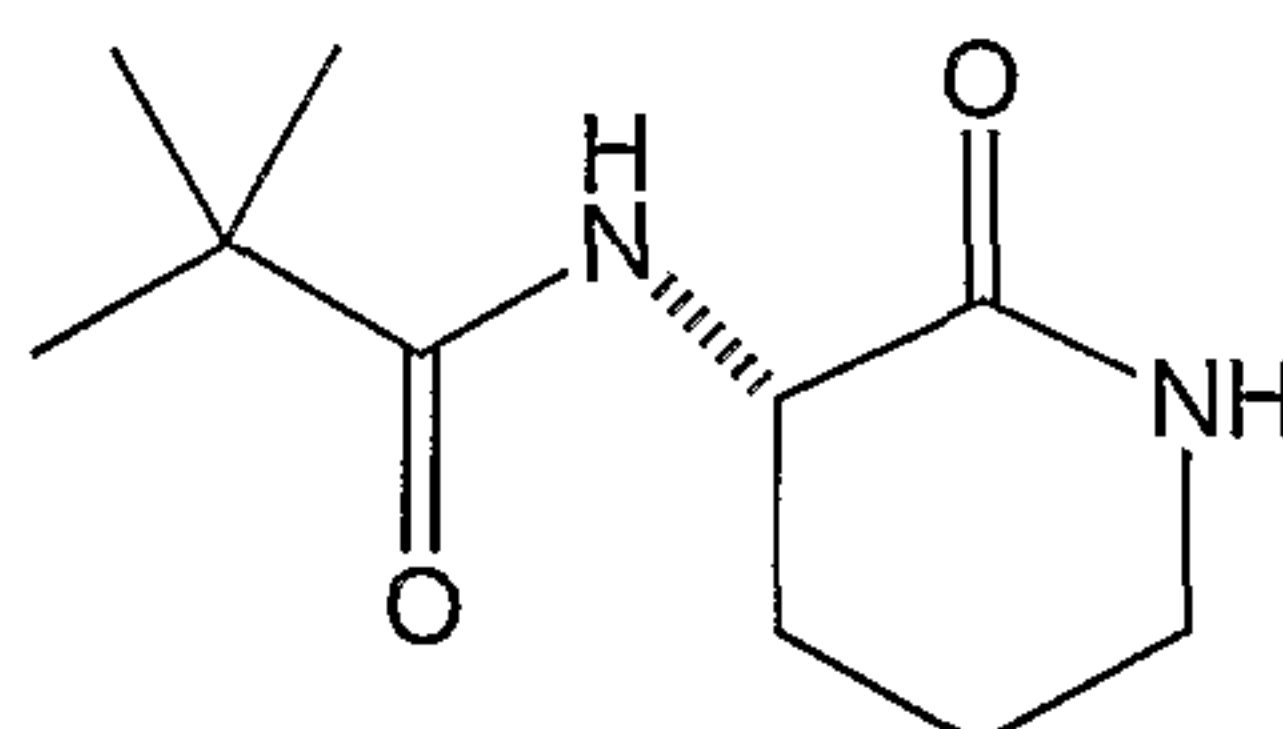
What is claimed is:

1. Use of a compound of formula (I) or a pharmaceutically acceptable salt thereof, for the preparation of a medicament intended to treat an inflammatory disorder:



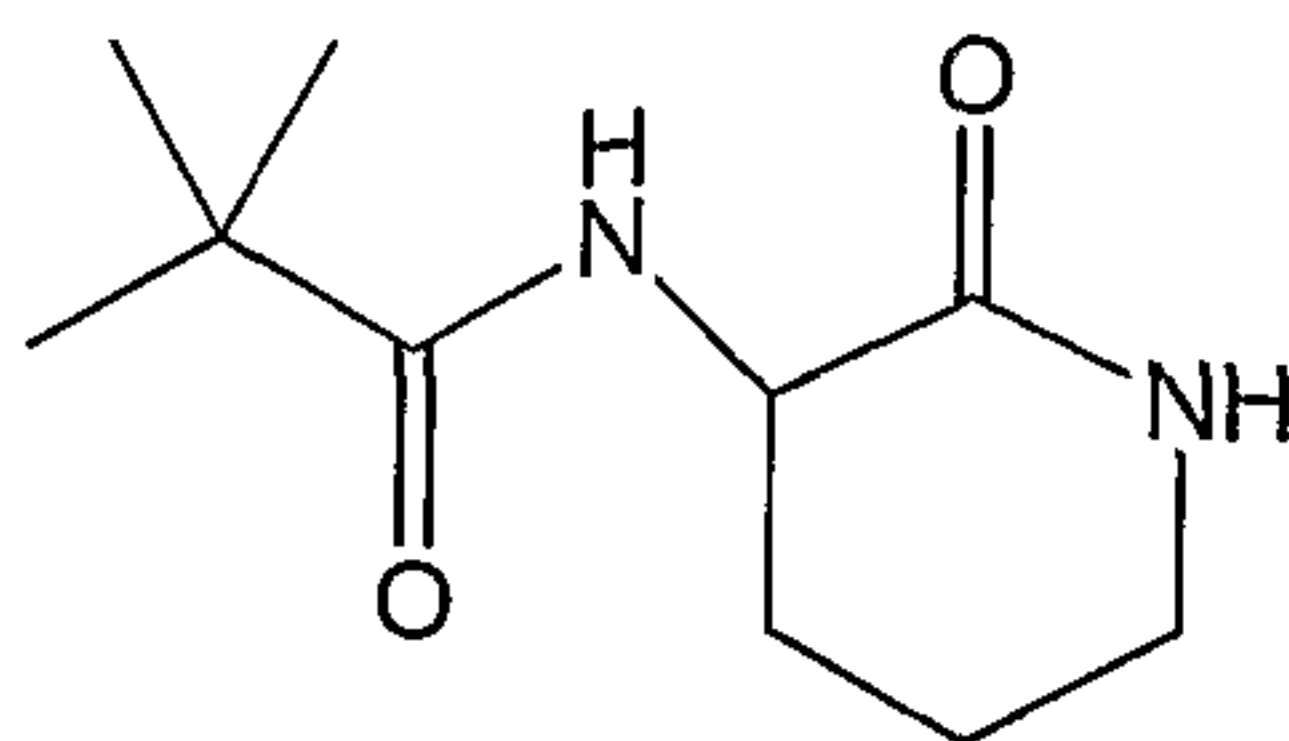
(I)

2. Use of a compound of formula (I') or a pharmaceutically acceptable salt thereof, for the preparation of a medicament intended to treat an inflammatory disorder:



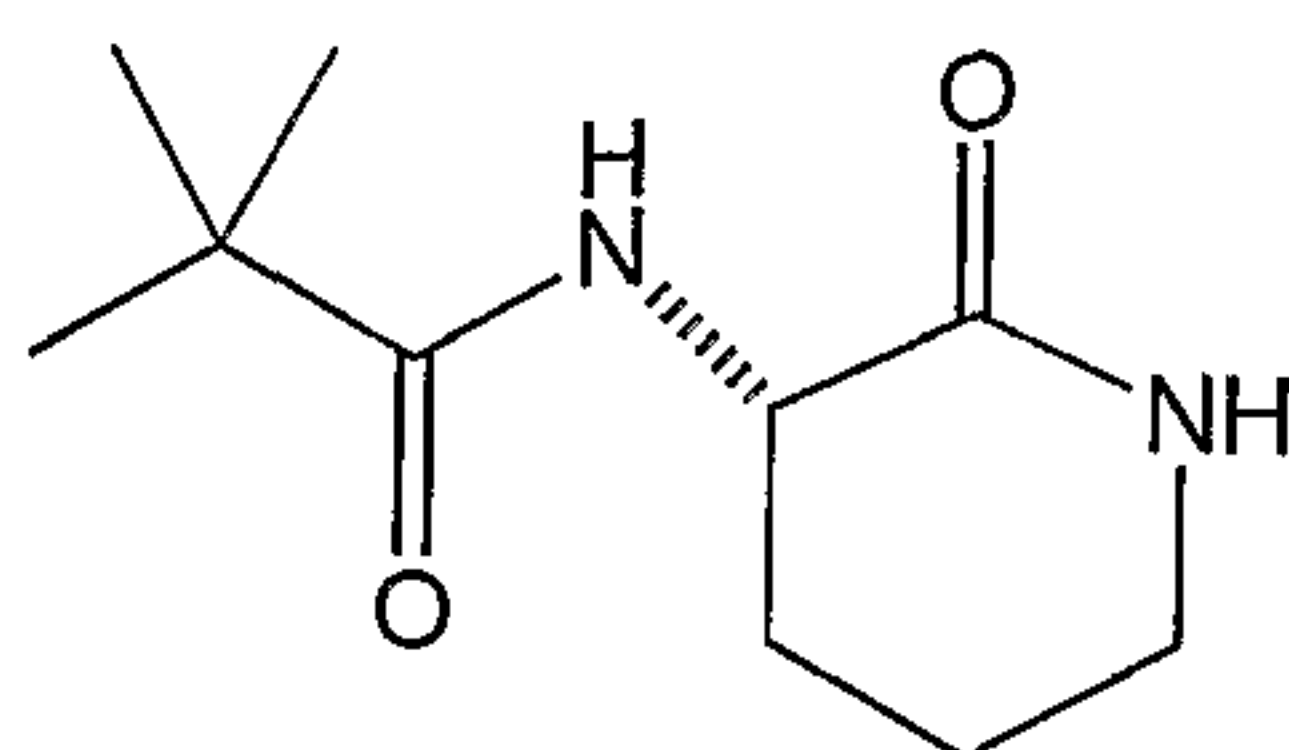
(I')

3. A pharmaceutically acceptable composition comprising, as active ingredient, a compound of formula (I) or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable excipient and/or carrier:



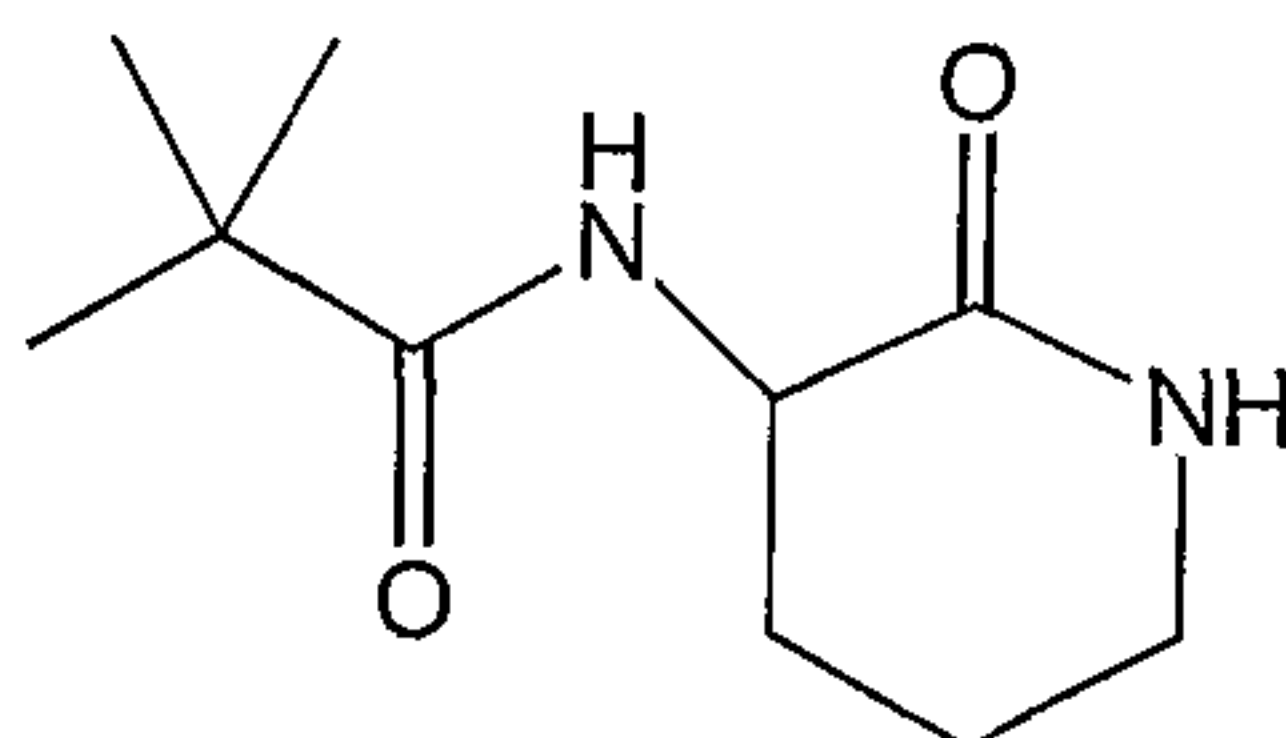
(I)

4. A pharmaceutically acceptable composition comprising, as active ingredient, a compound of formula (I') or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable excipient and/or carrier:



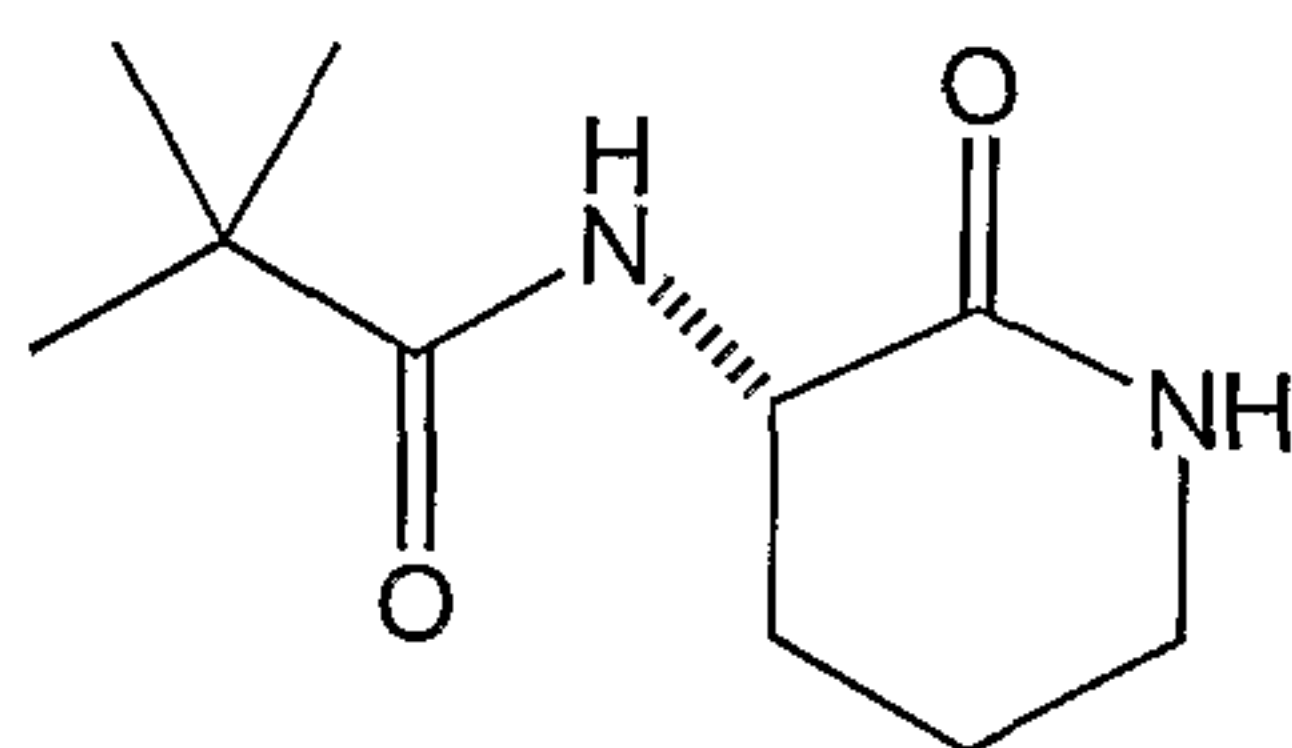
(I')

5. A compound of general formula (I):



(I)

6. A compound of general formula (I'):



(I')

7. Use of a compound of formula (I) or (I') according to one of claims 1 or 2 wherein the inflammatory disorder is selected from the group consisting of autoimmune

diseases, vascular disorders, viral infection or replication, asthma, osteoporosis (low bone mineral density), tumor growth, organ transplant rejection and/or delayed graft or organ function, a disorder characterised by an elevated TNF- α level, psoriasis, skin wounds, disorders caused by intracellular parasites, neuropathic pain, allergies,

5 Alzheimer's disease, antigen induced recall response, immune response suppression, rheumatoid arthritis, multiple sclerosis, ALS, fibrosis, and formation of adhesions.

8. Use of a compound of formula (I) or (I') according to claim 7 where the inflammatory disorder is asthma, allergic rhinitis or chronic obstructive pulmonary disease.

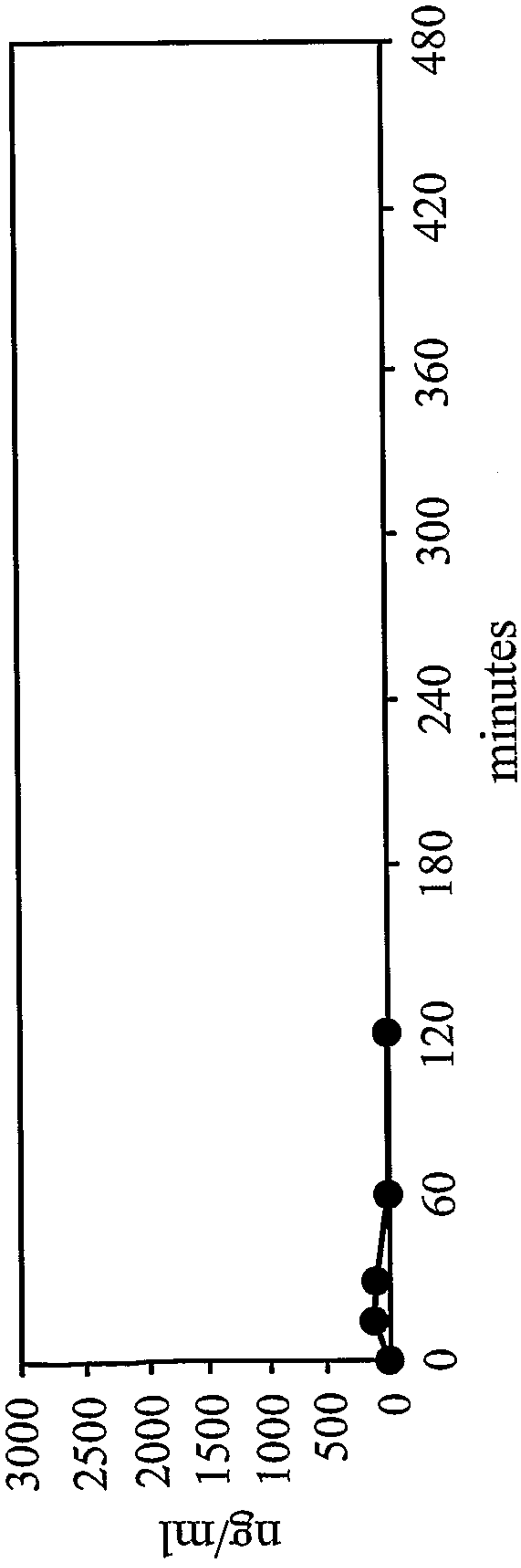
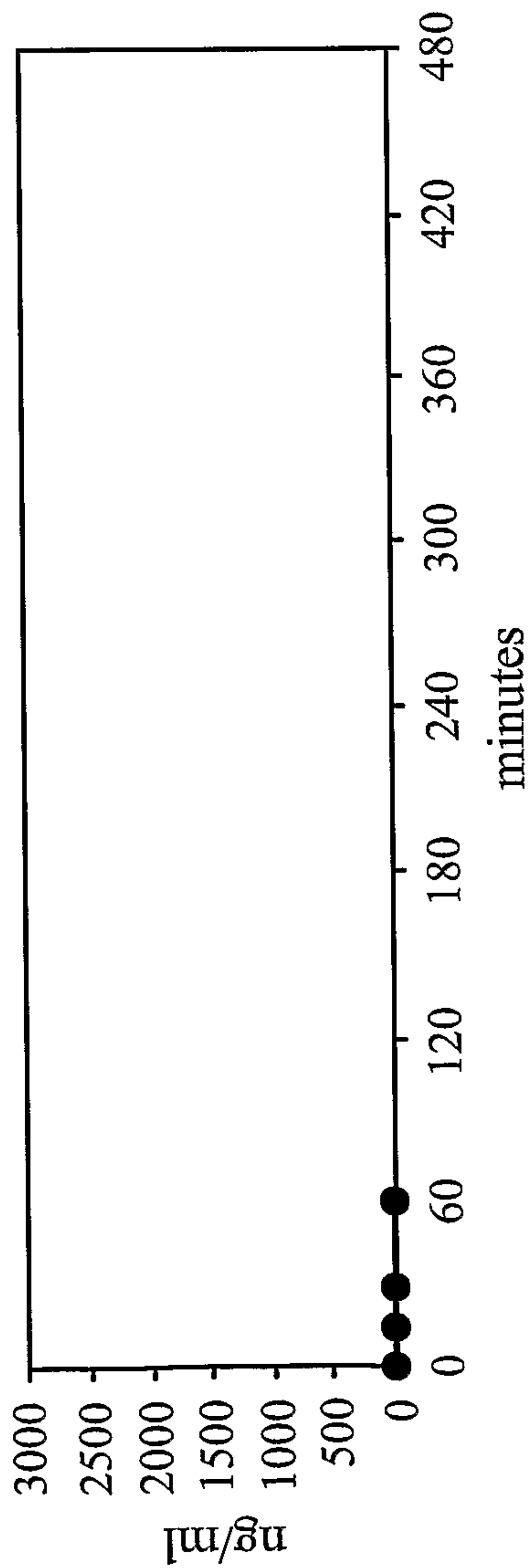
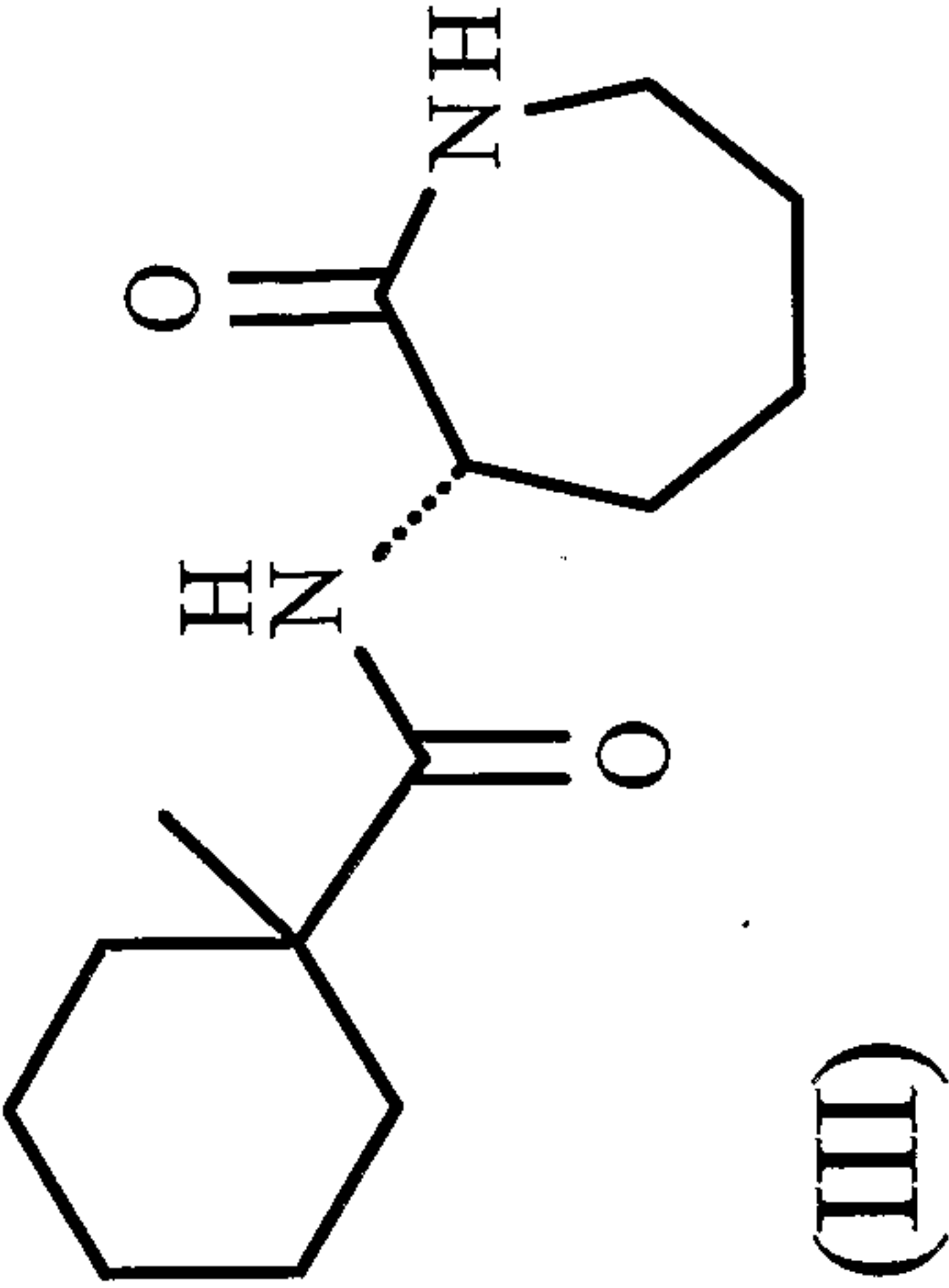
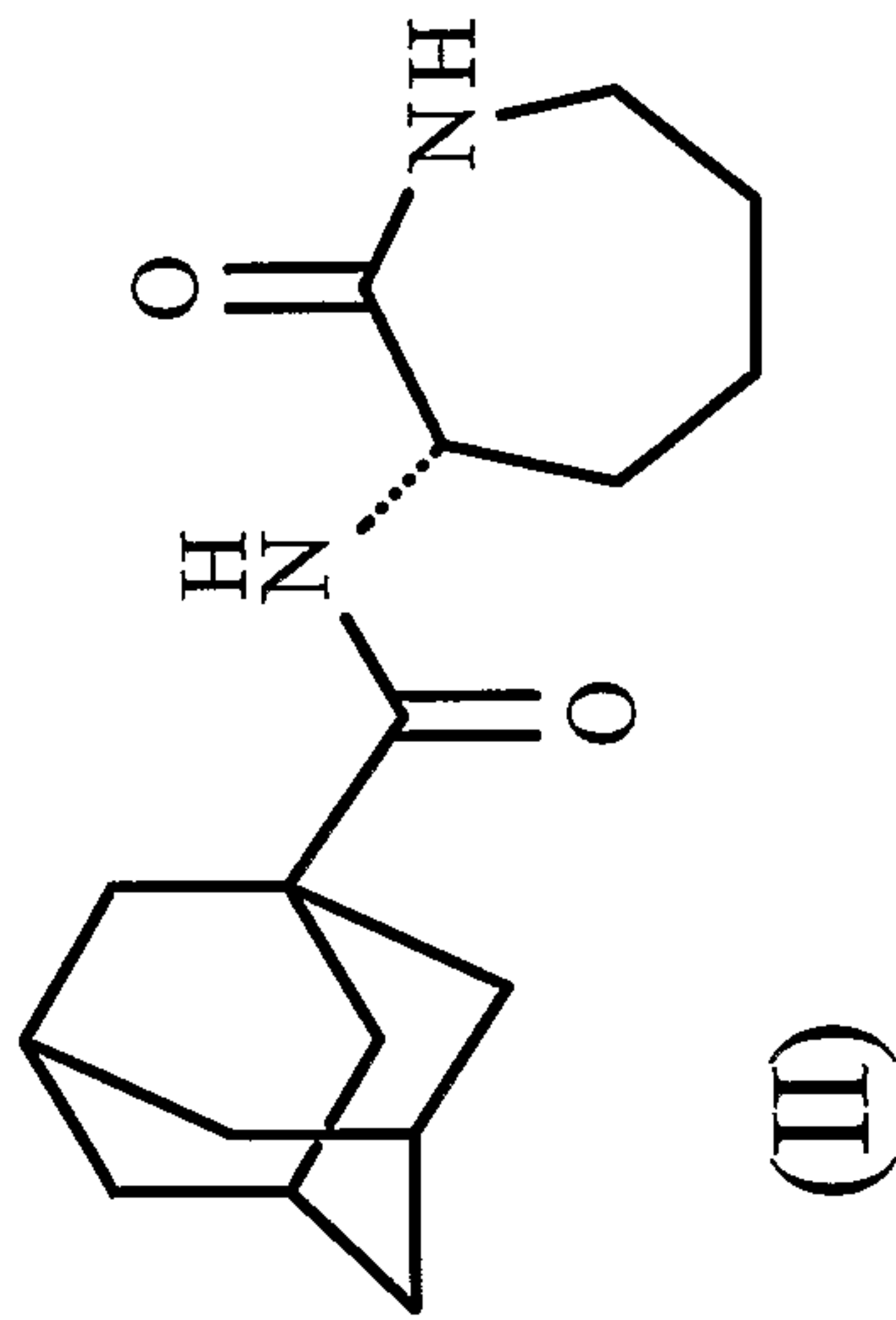
10 9. Use of a compound of formula (I) or (I') according to claim 7 where the inflammatory disorder is the formation of adhesions following gynaecological or general surgery.

10. Use of a compound of formula (I) or (I') according to claim 7 where the inflammatory disorder is nephropathy, including diabetic nephropathy.

15 11. A method of treatment, amelioration or prophylaxis of the symptoms of an inflammatory disease (including an adverse inflammatory reaction to any agent) by the administration to a patient of an anti-inflammatory amount of a compound, composition or medicament as defined in any of claims 1 to 10.

20 12. A pharmaceutically acceptable composition according to claim 3 or 4, for use in treatment, amelioration or prophylaxis of the symptoms of an inflammatory disorder.

13. A pharmaceutically acceptable composition according to claim 12, wherein the disorder is selected from the disorders defined in any of claims 7 to 10.



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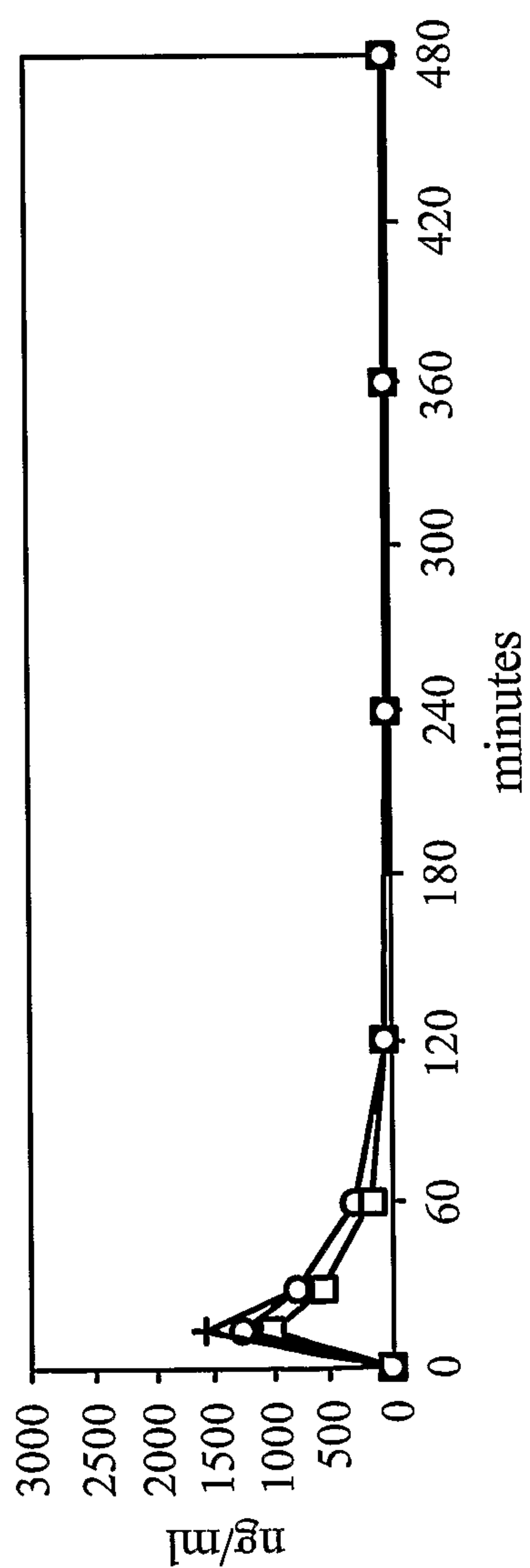
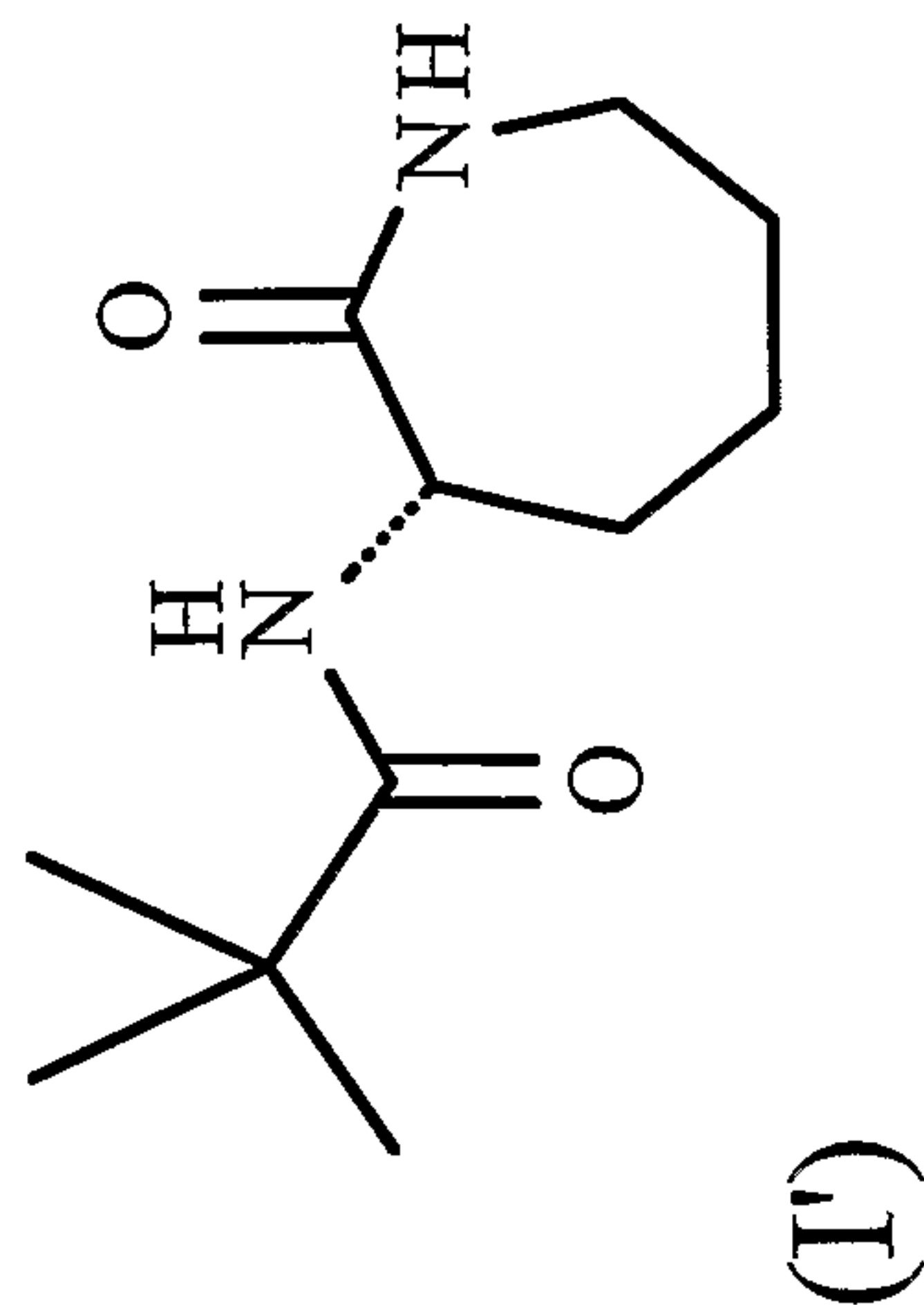
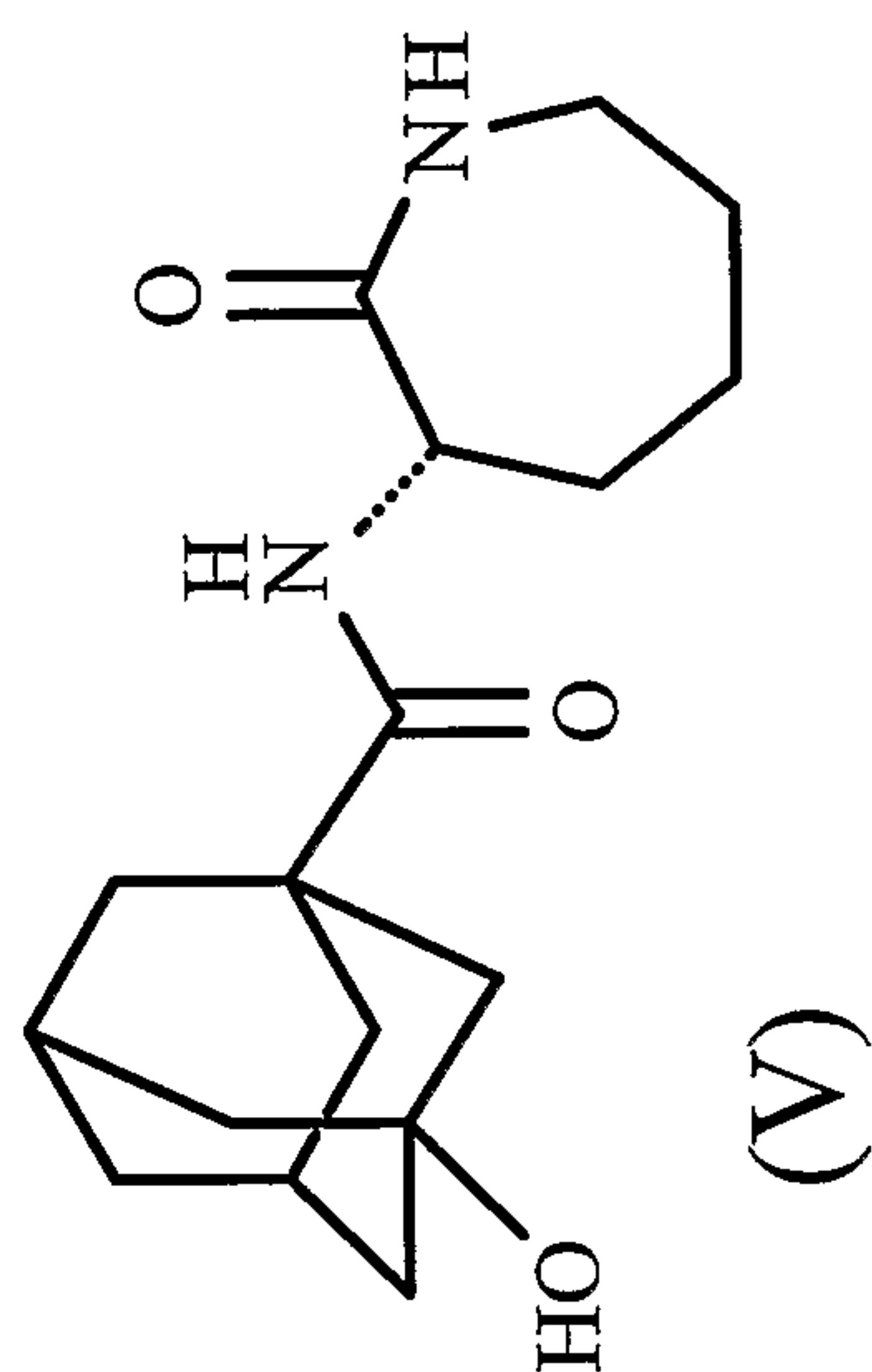


FIG. 1C

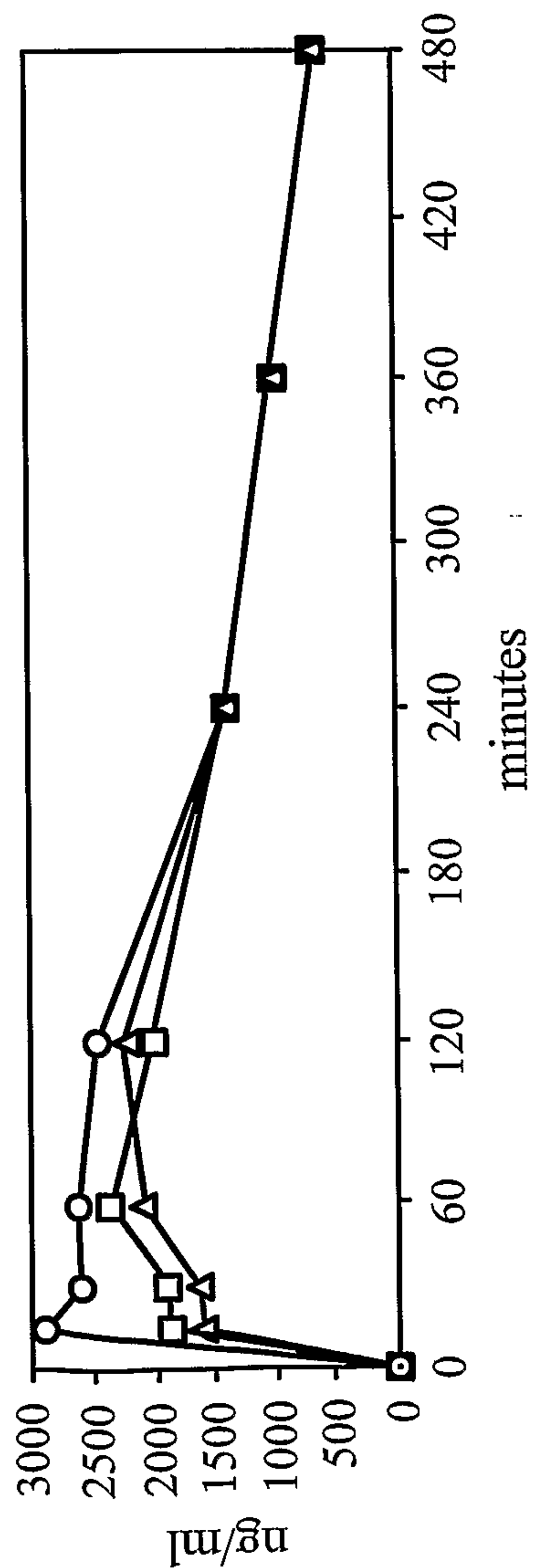
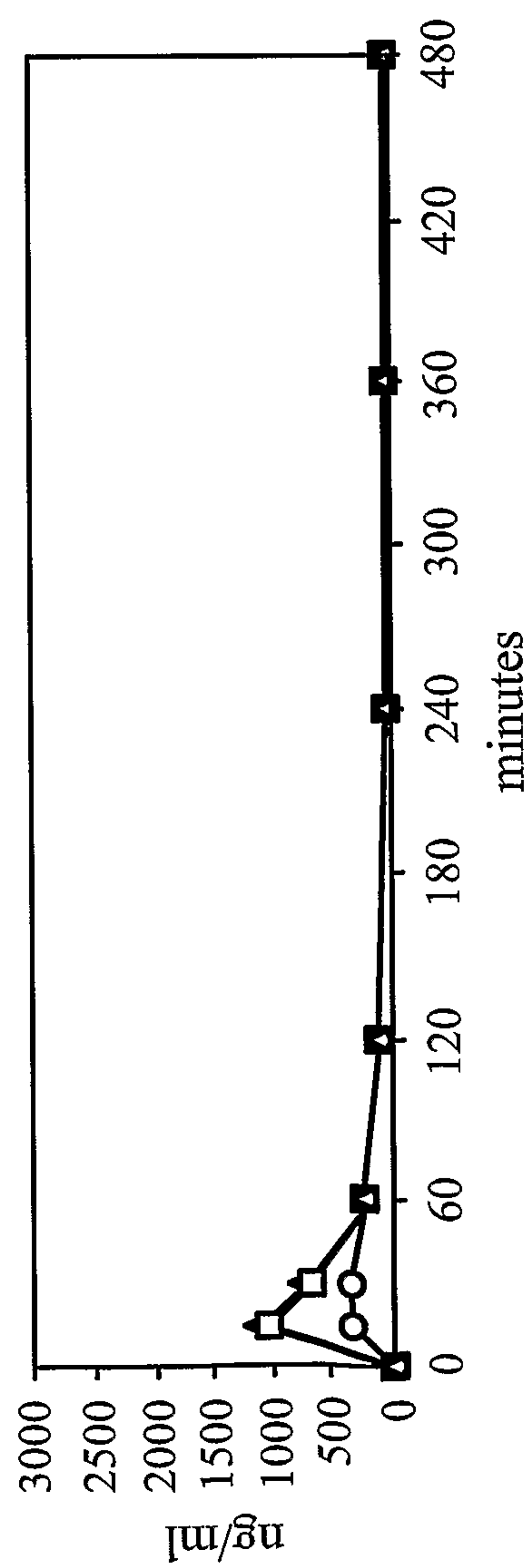
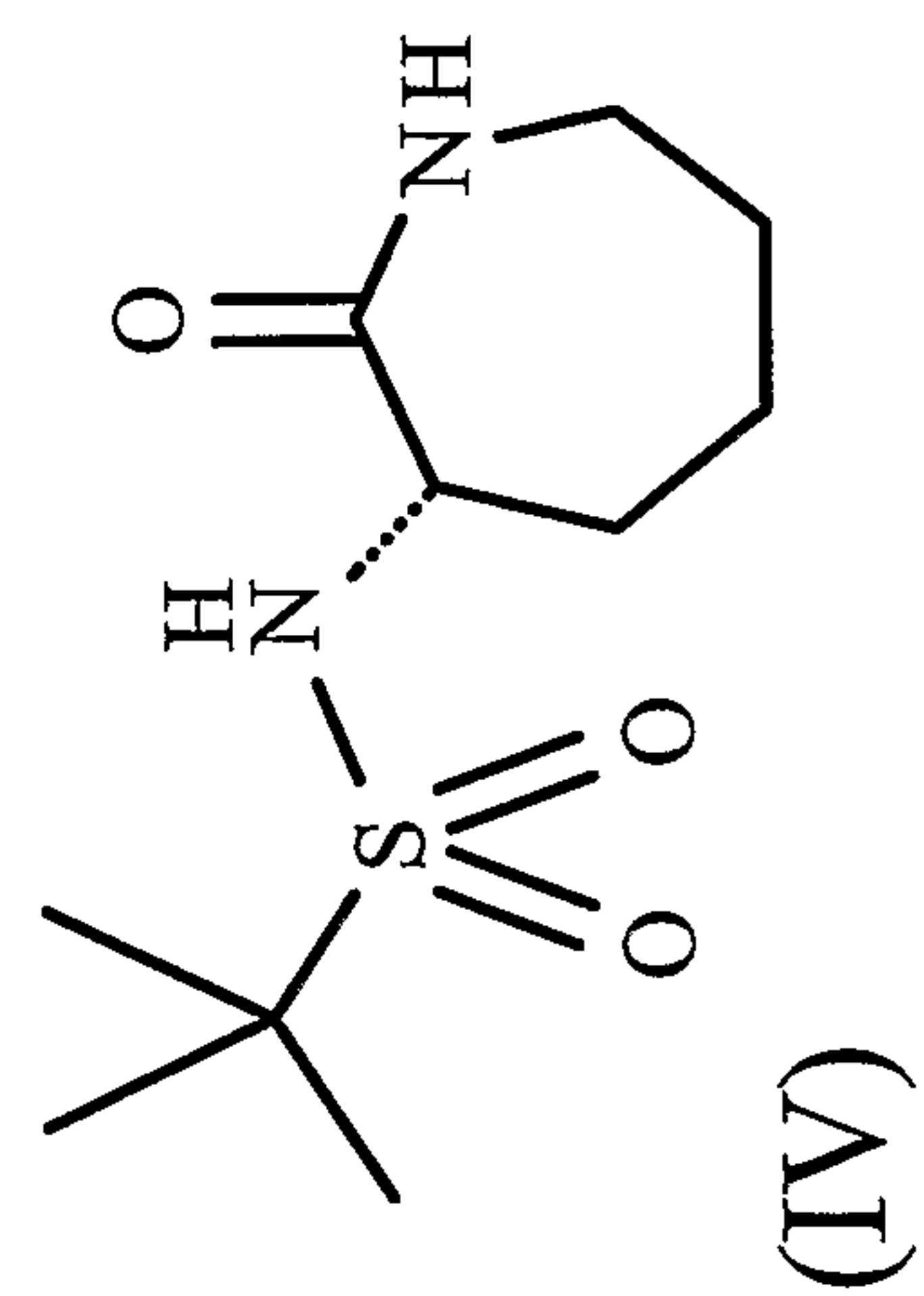


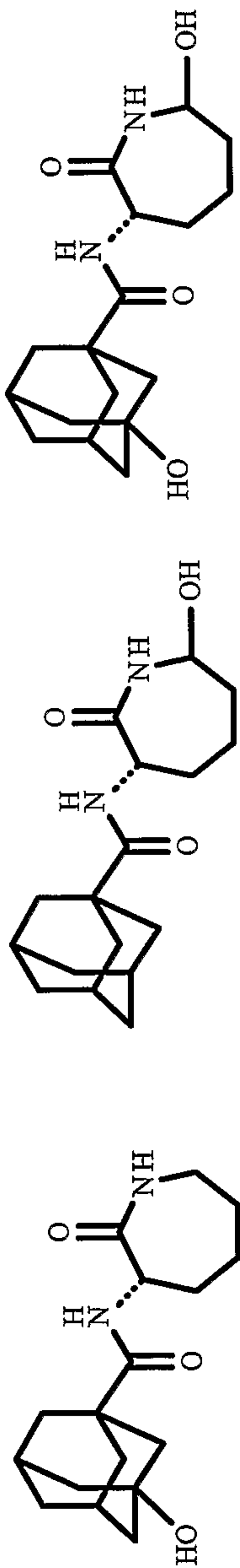
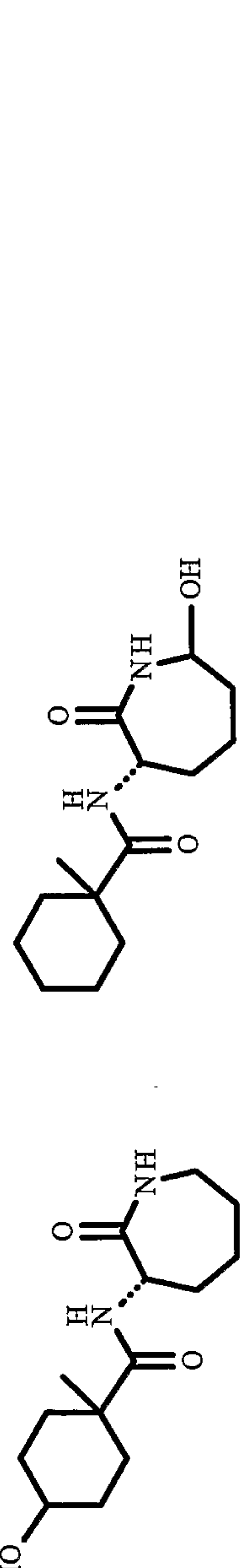
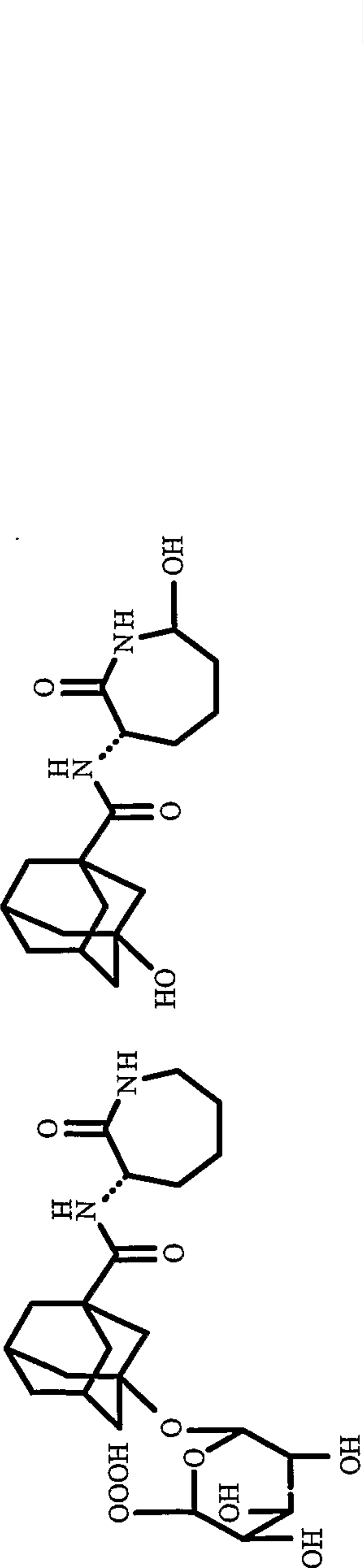
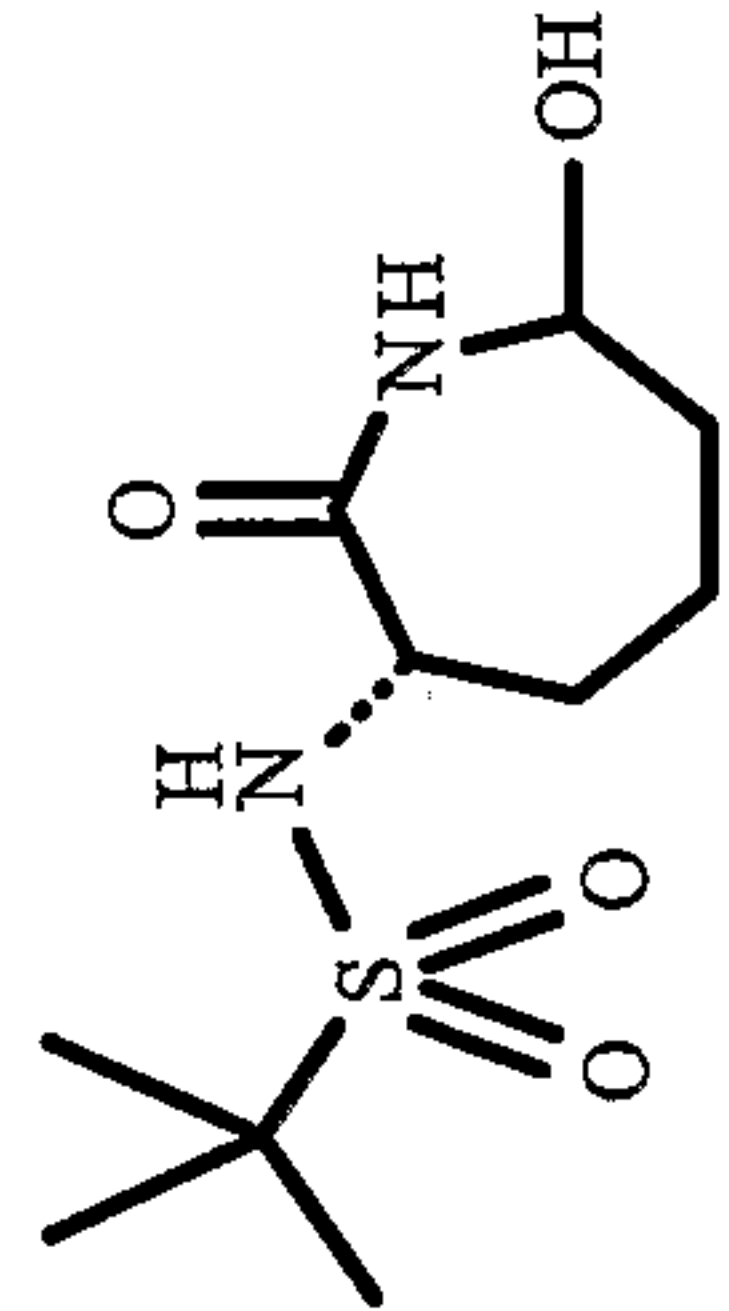
FIG. 1D

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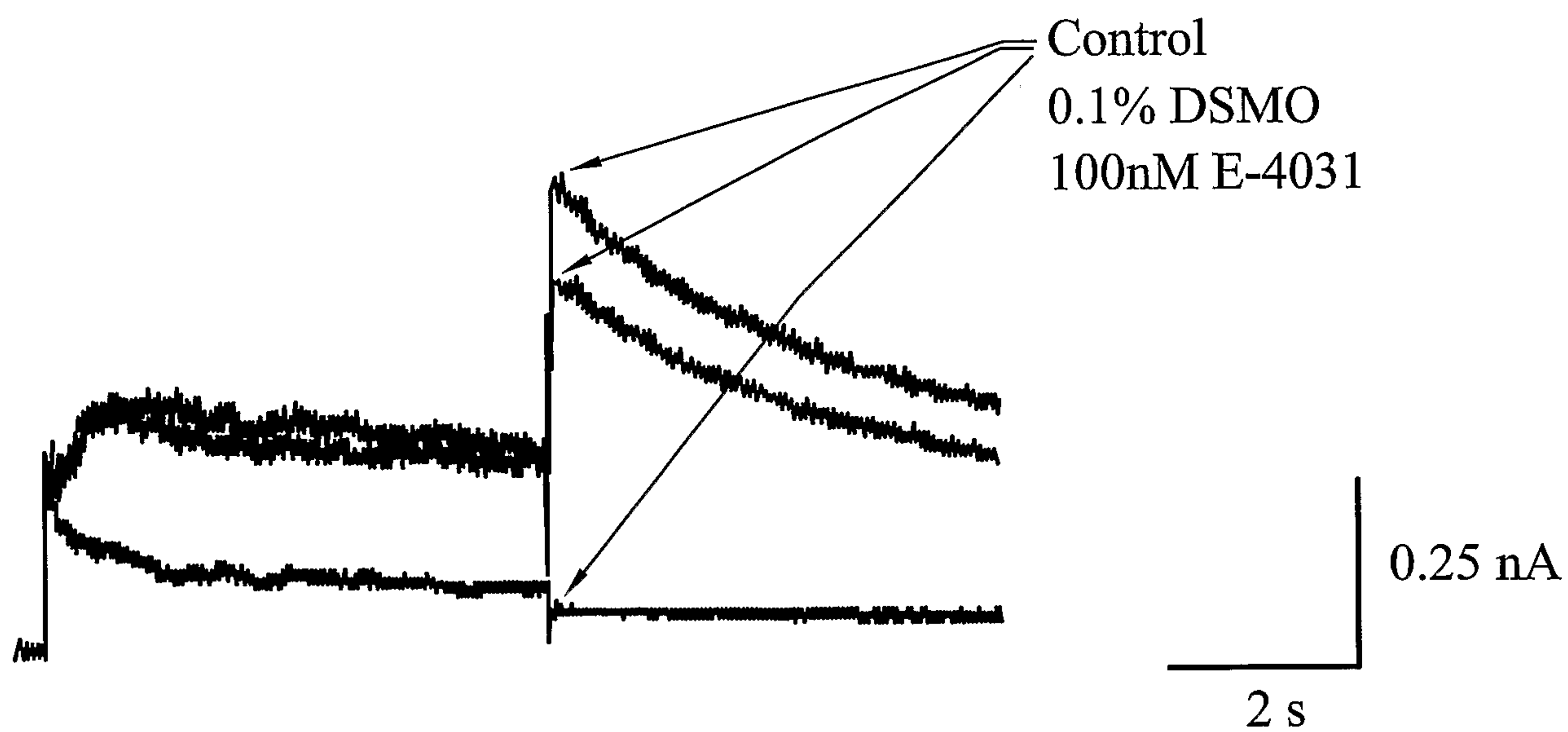
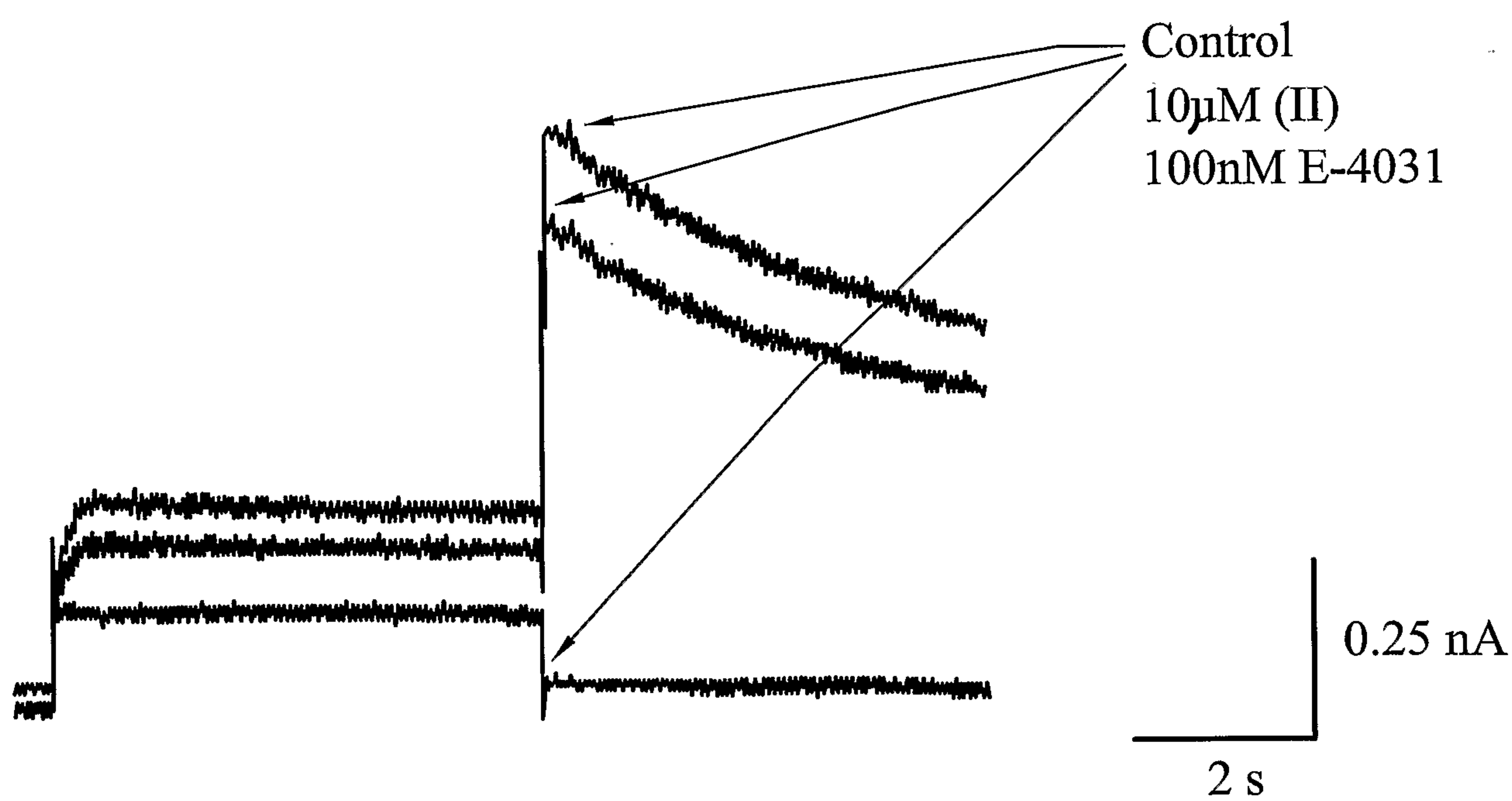
FIG. 1E

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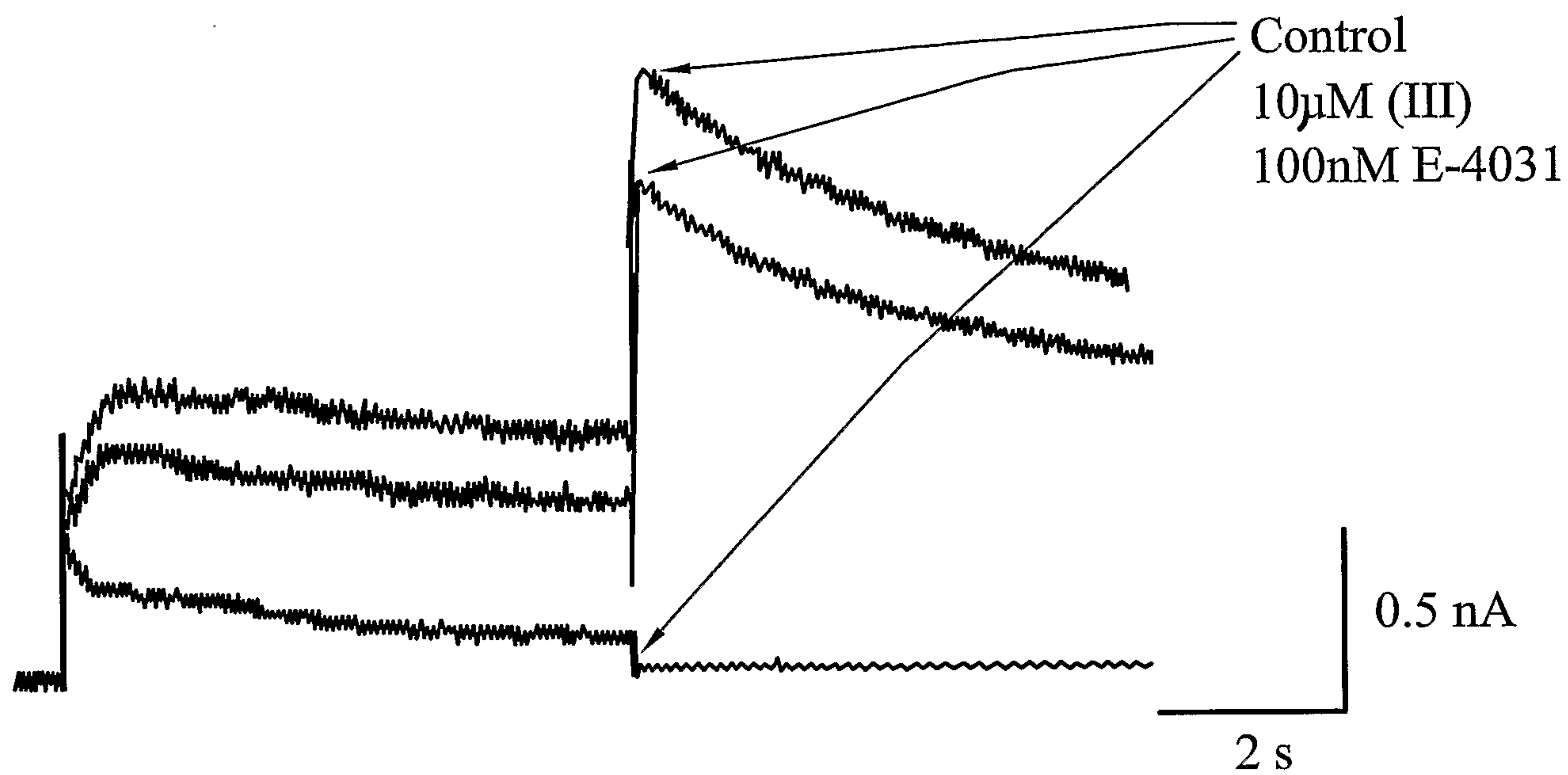
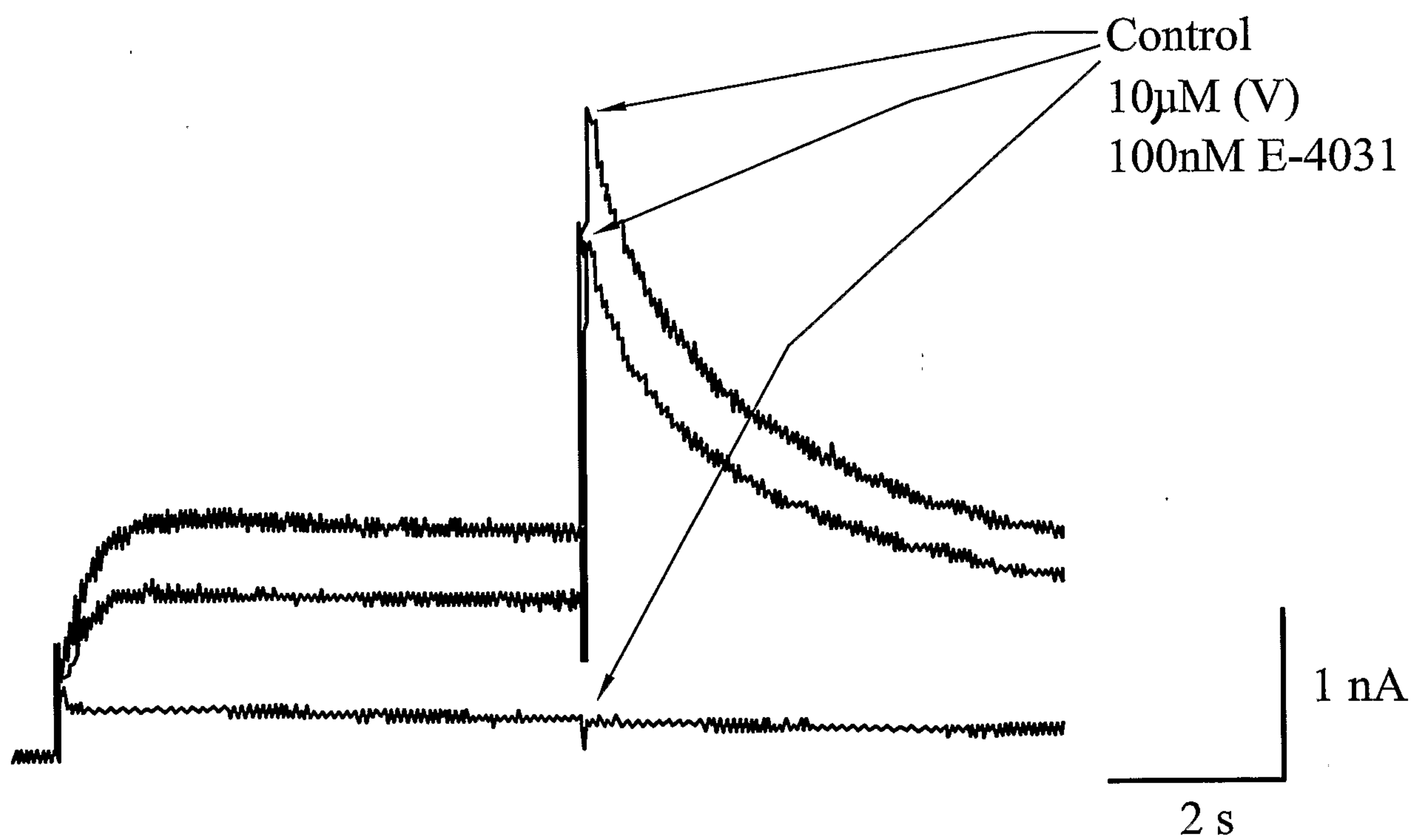
FIG. 2

(II)	
(III)	
(V)	
(I')	None
(IV)	Structure Unknown 

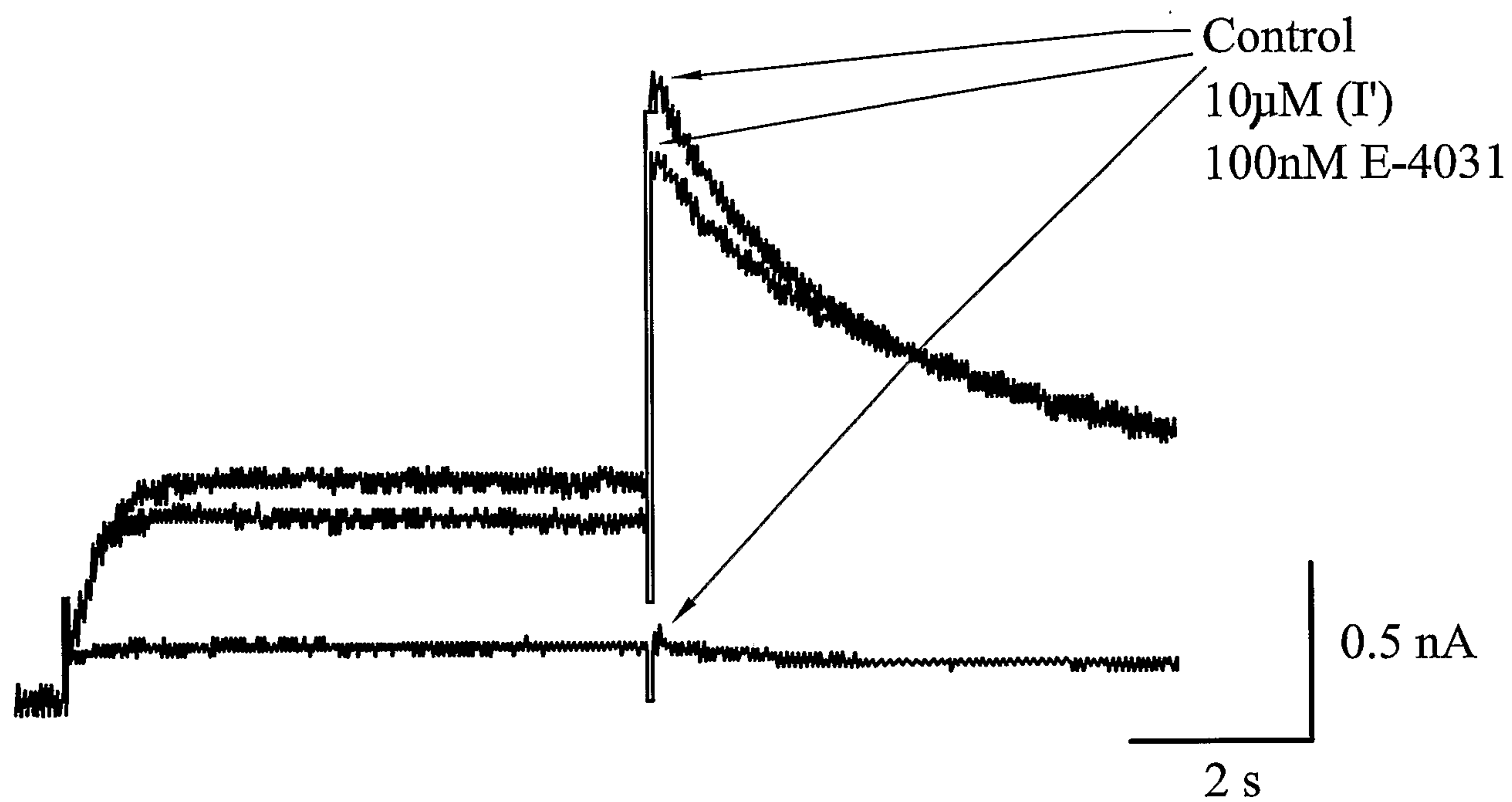
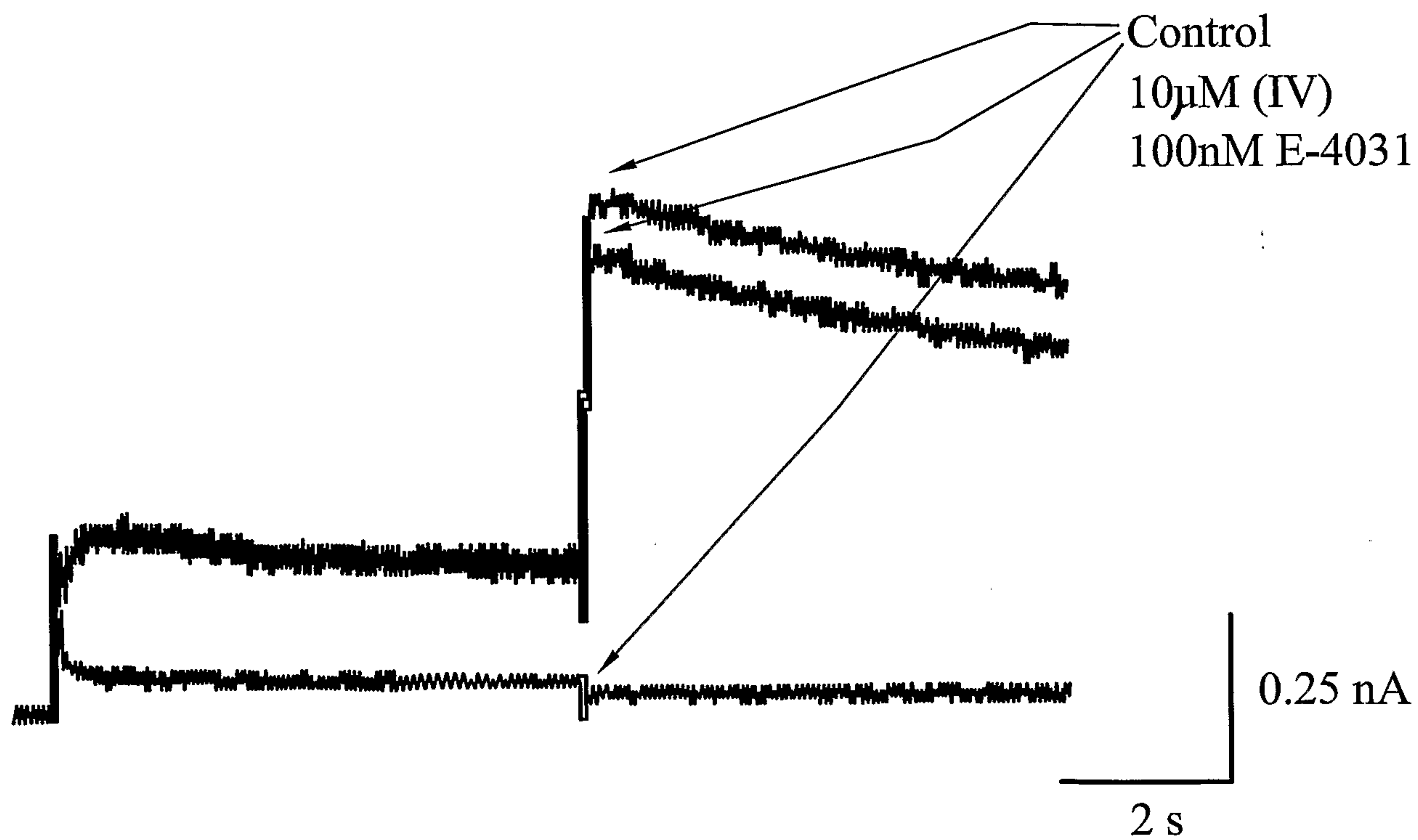
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FIG. 3AFIG. 3B

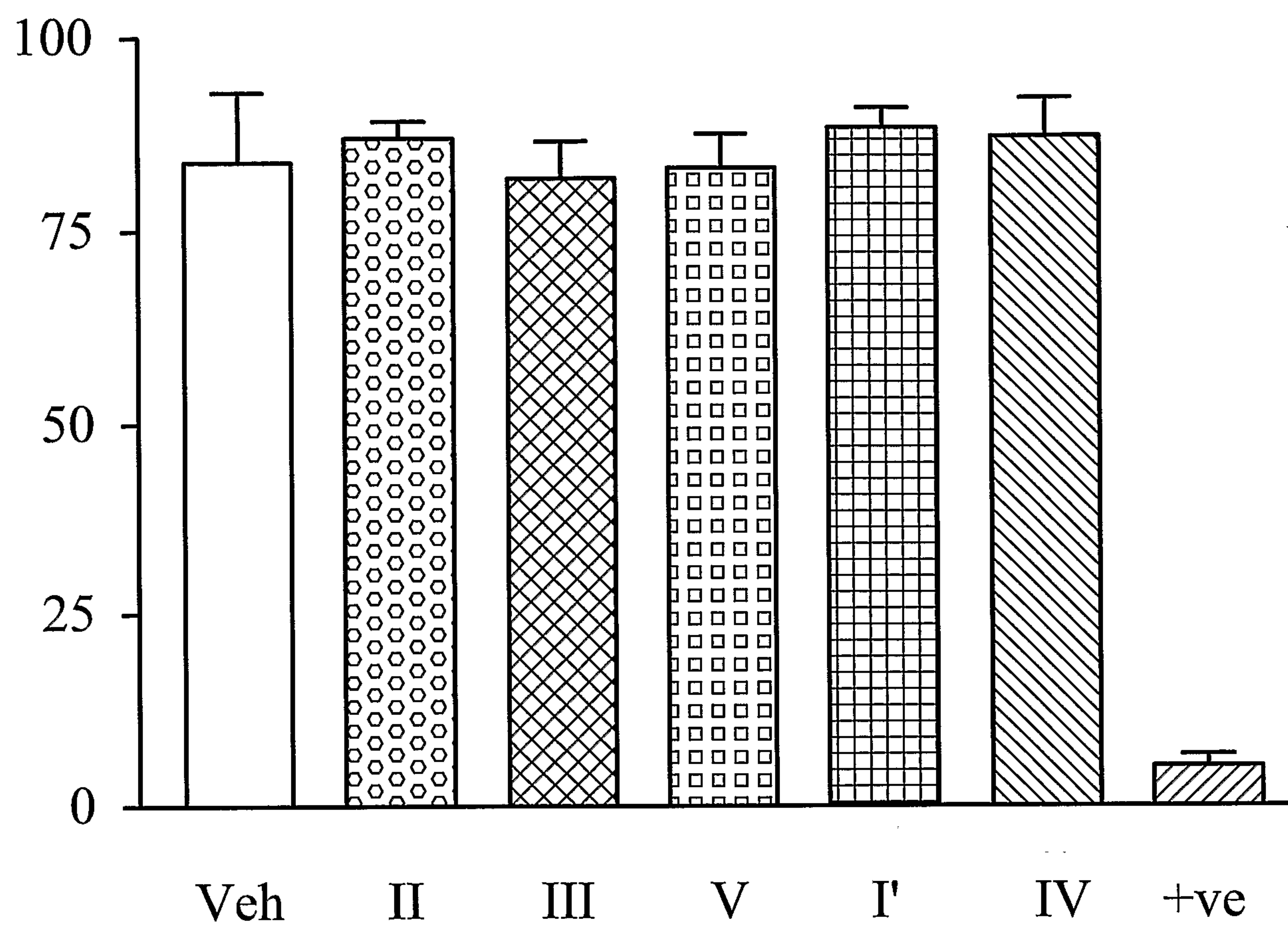
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FIG. 3CFIG. 3D

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FIG. 3EFIG. 3F

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FIG. 3G

-9/21-

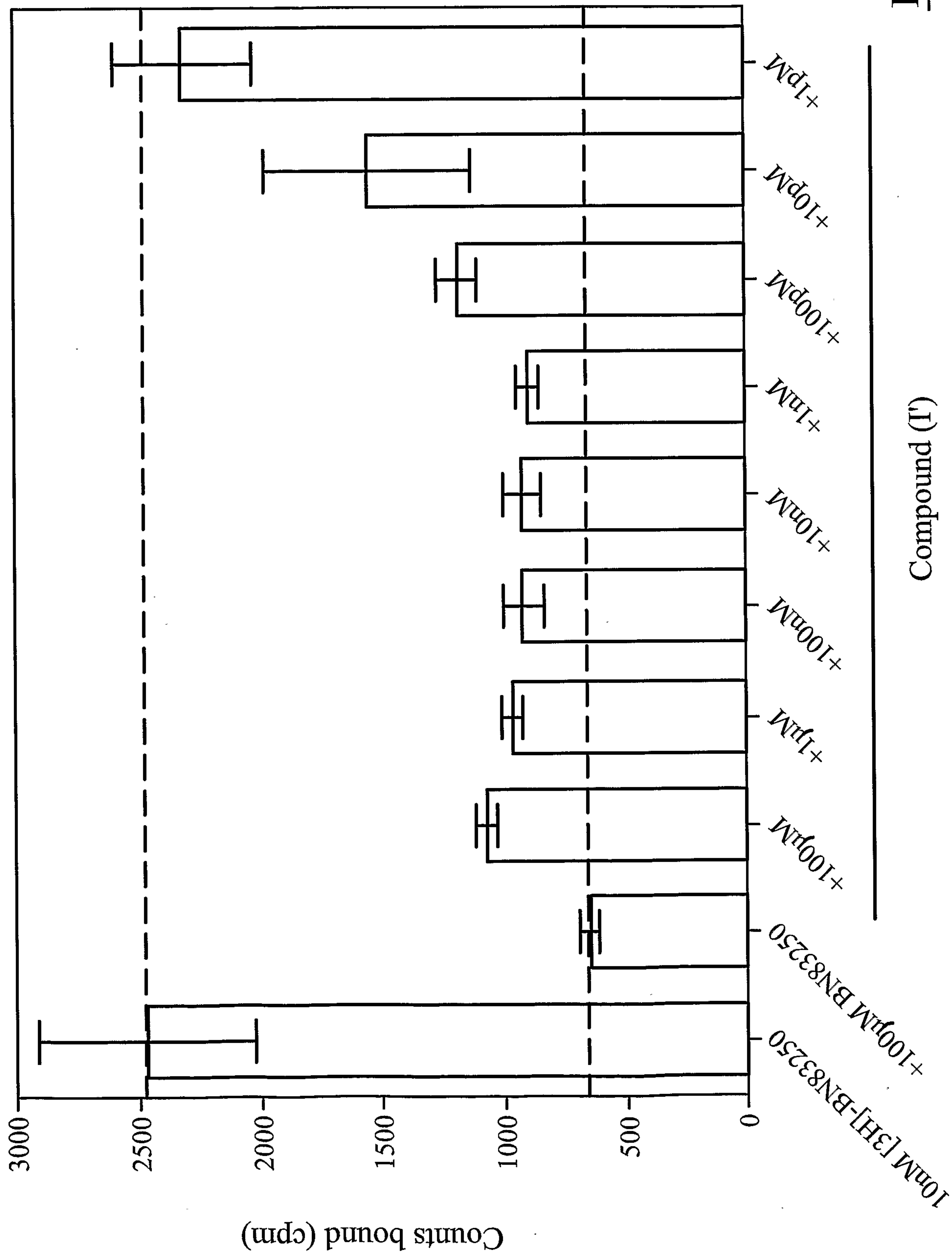
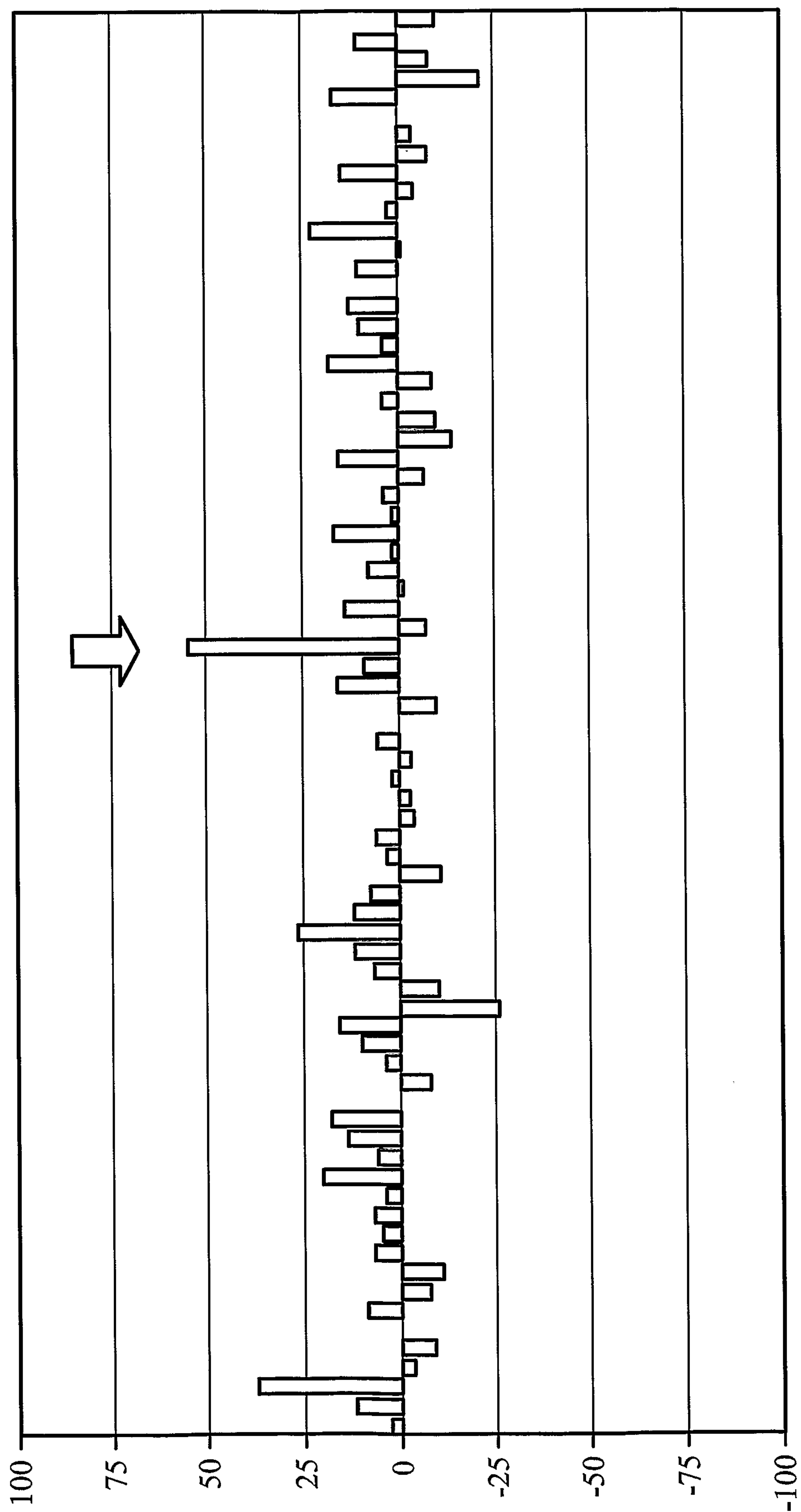
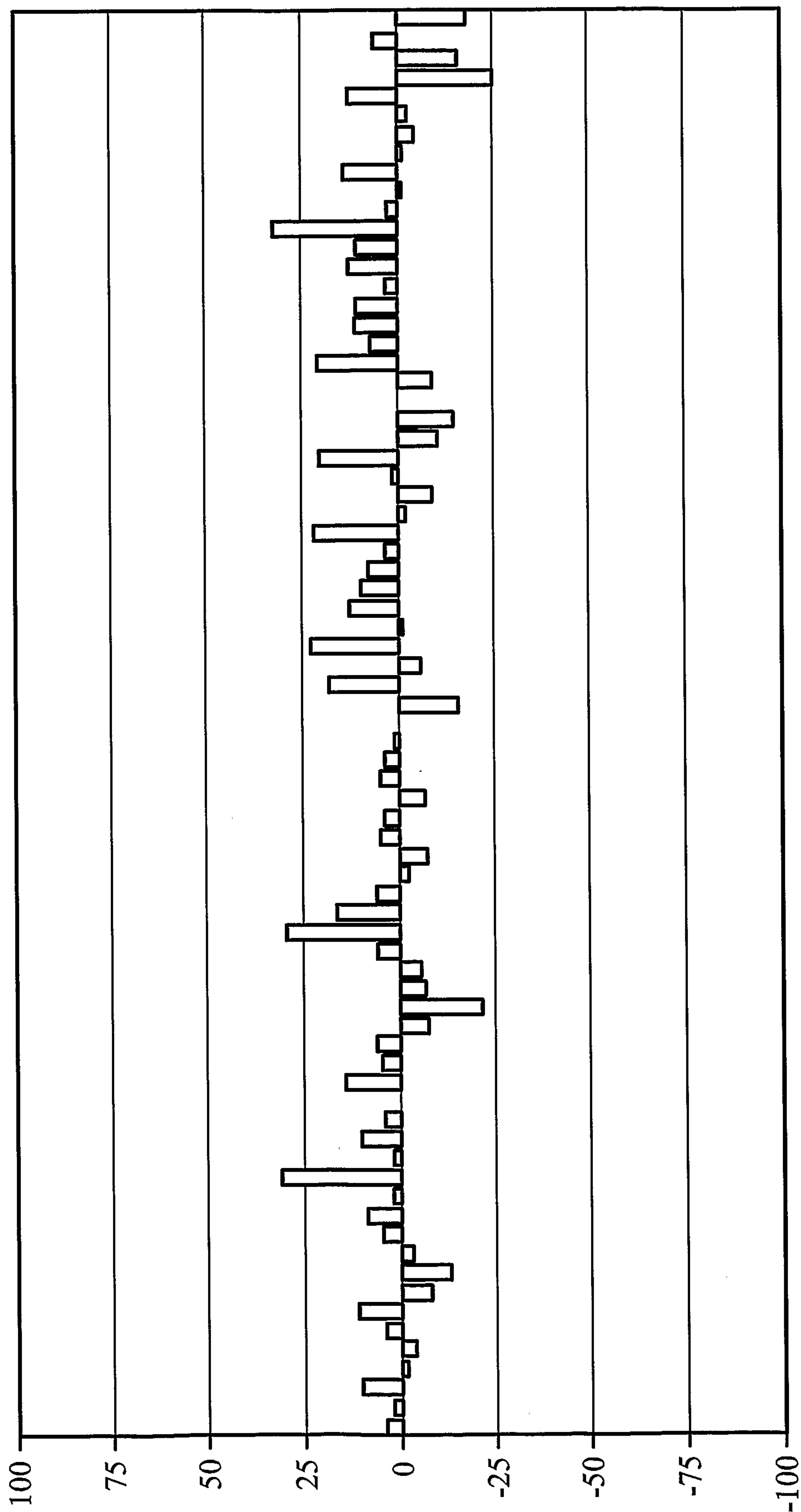


FIG. 4

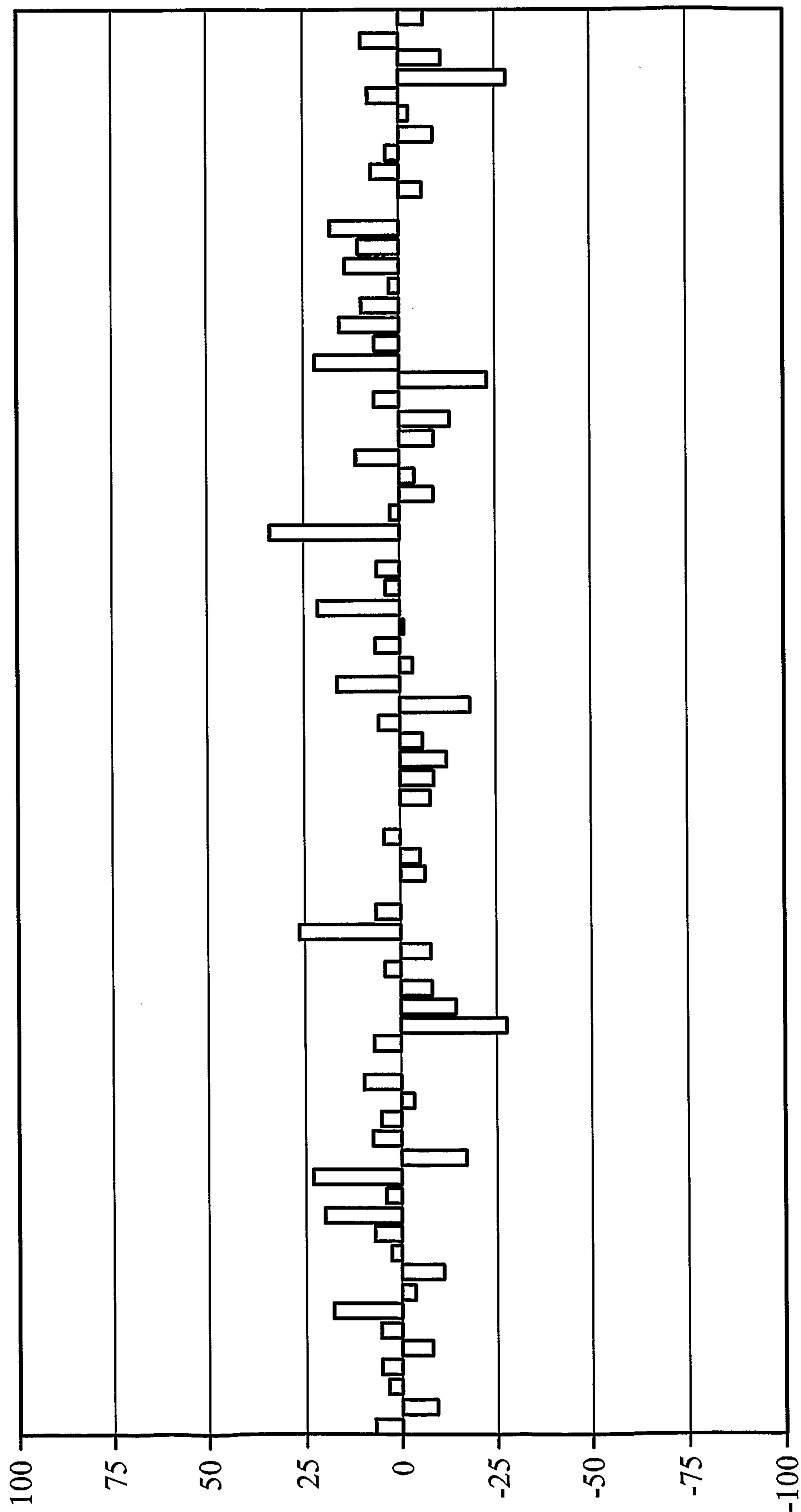
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FIG. 5A

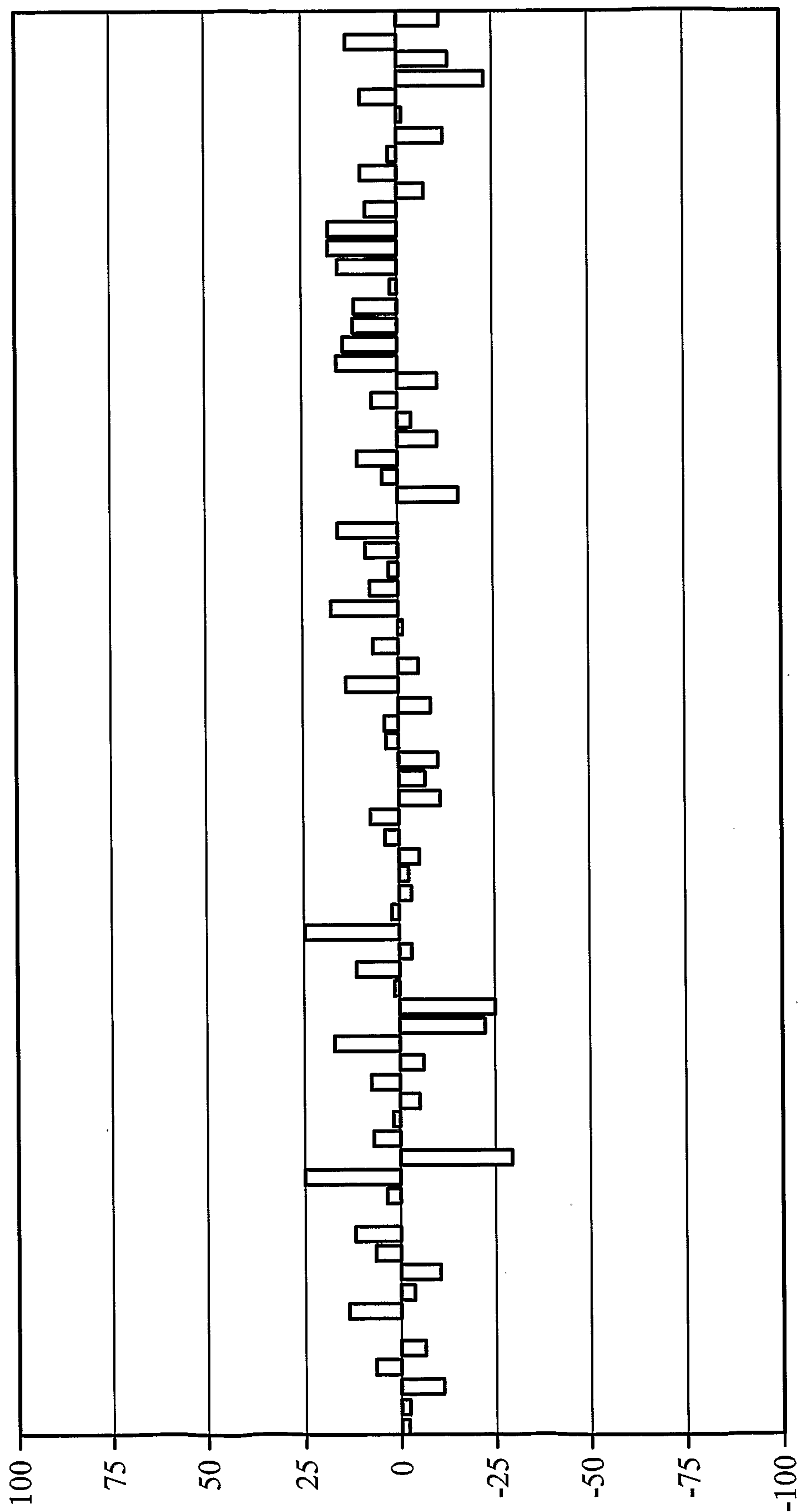
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FIG. 5B

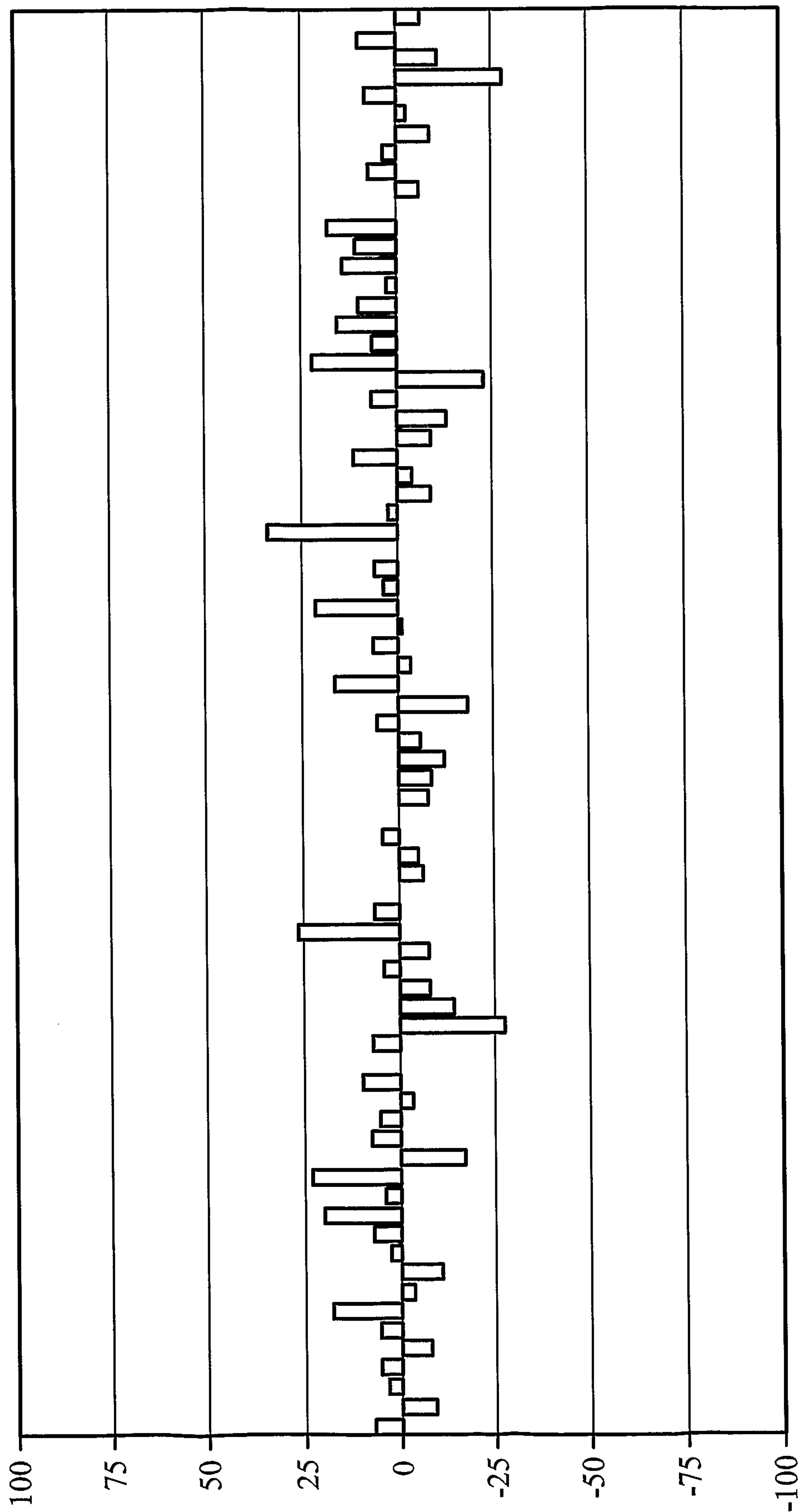
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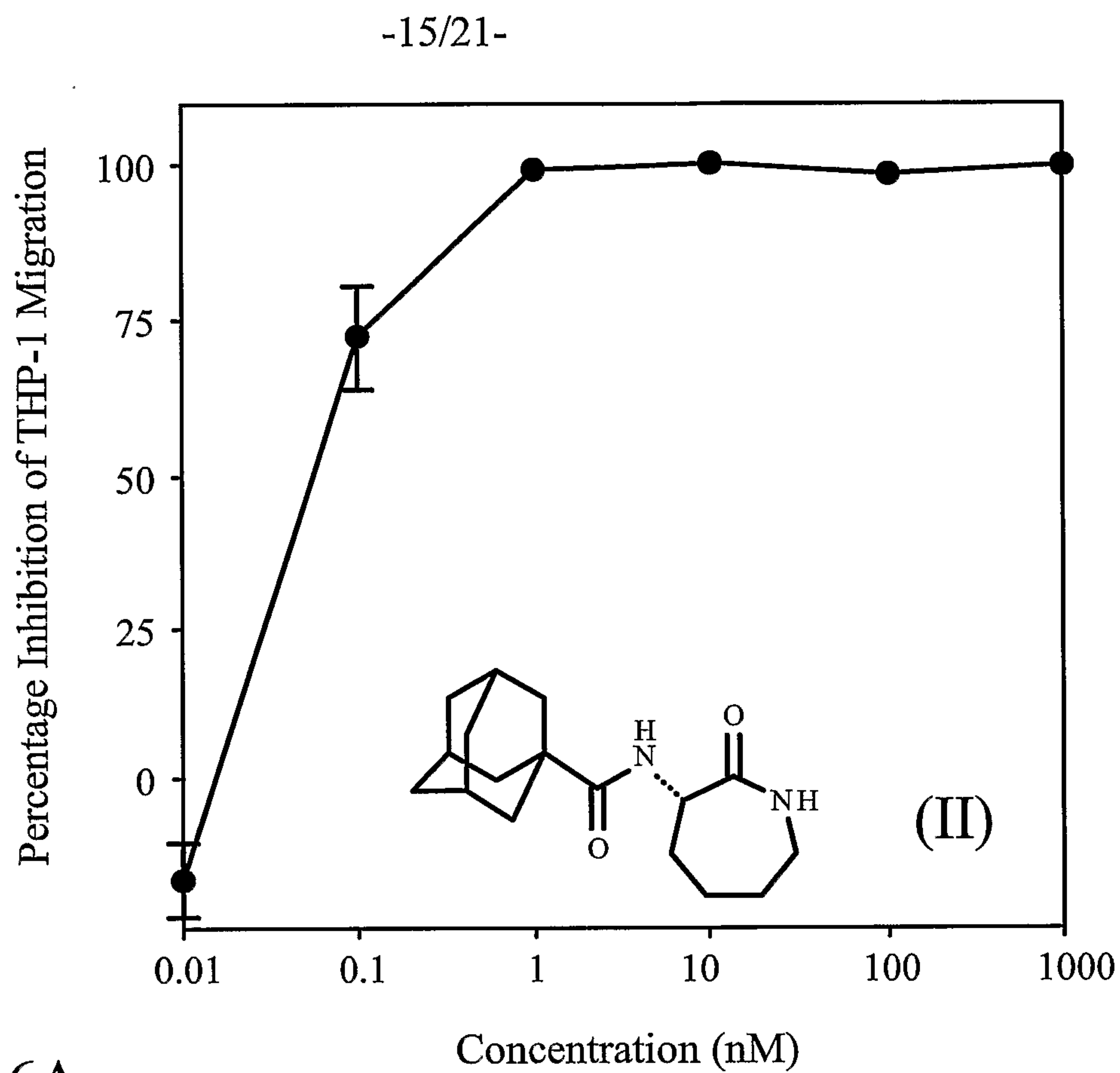
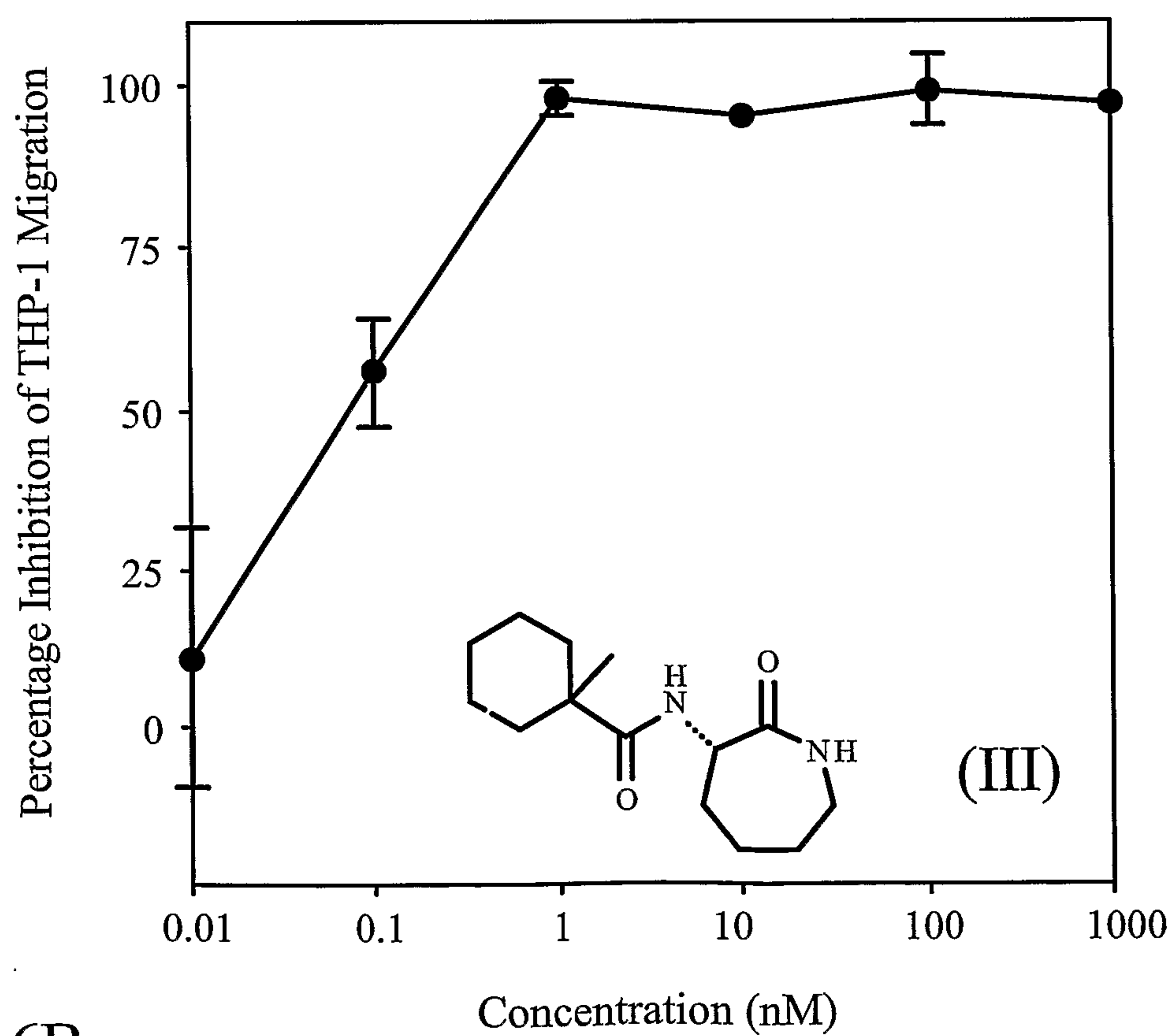
FIG. 5C

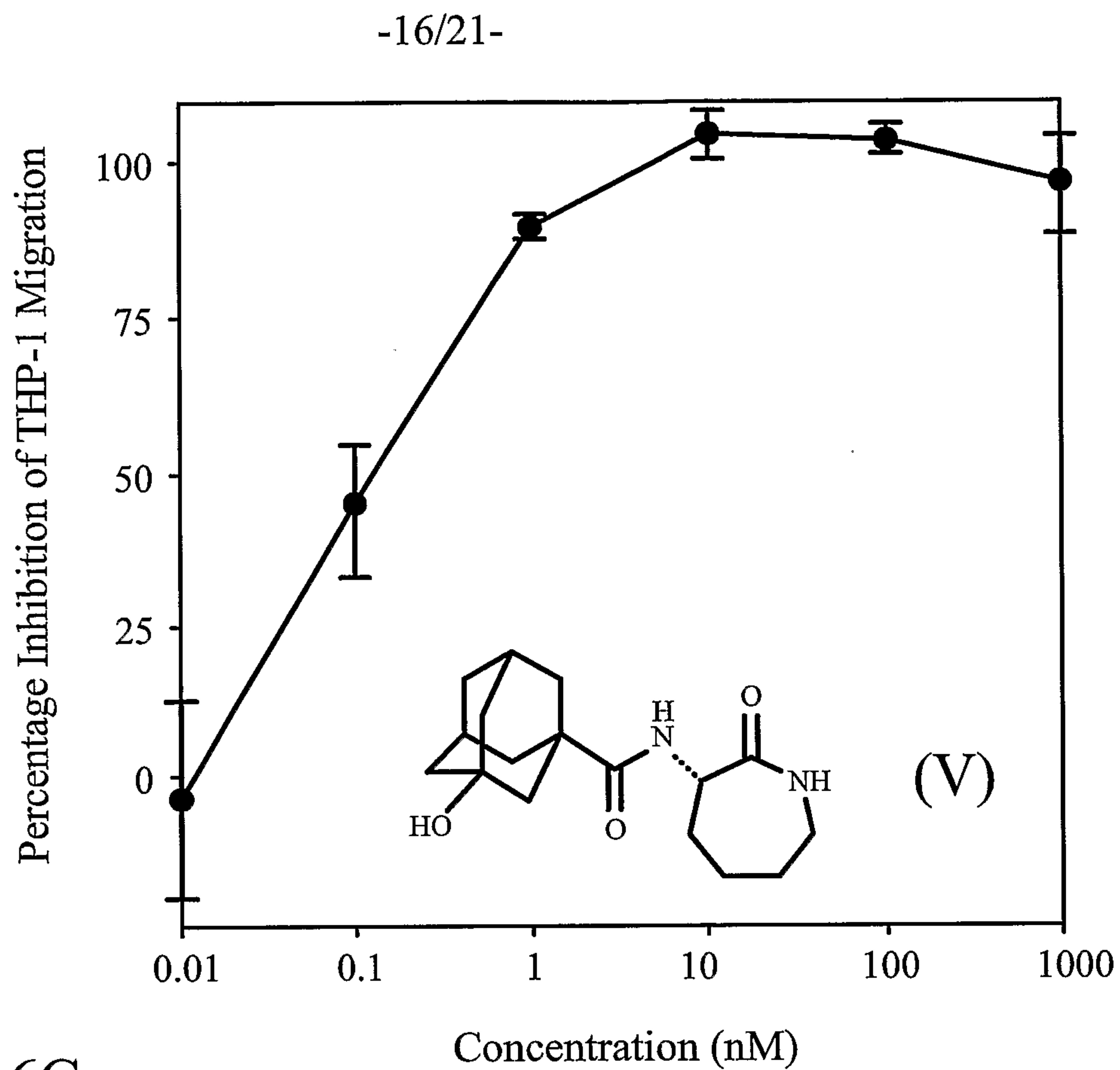
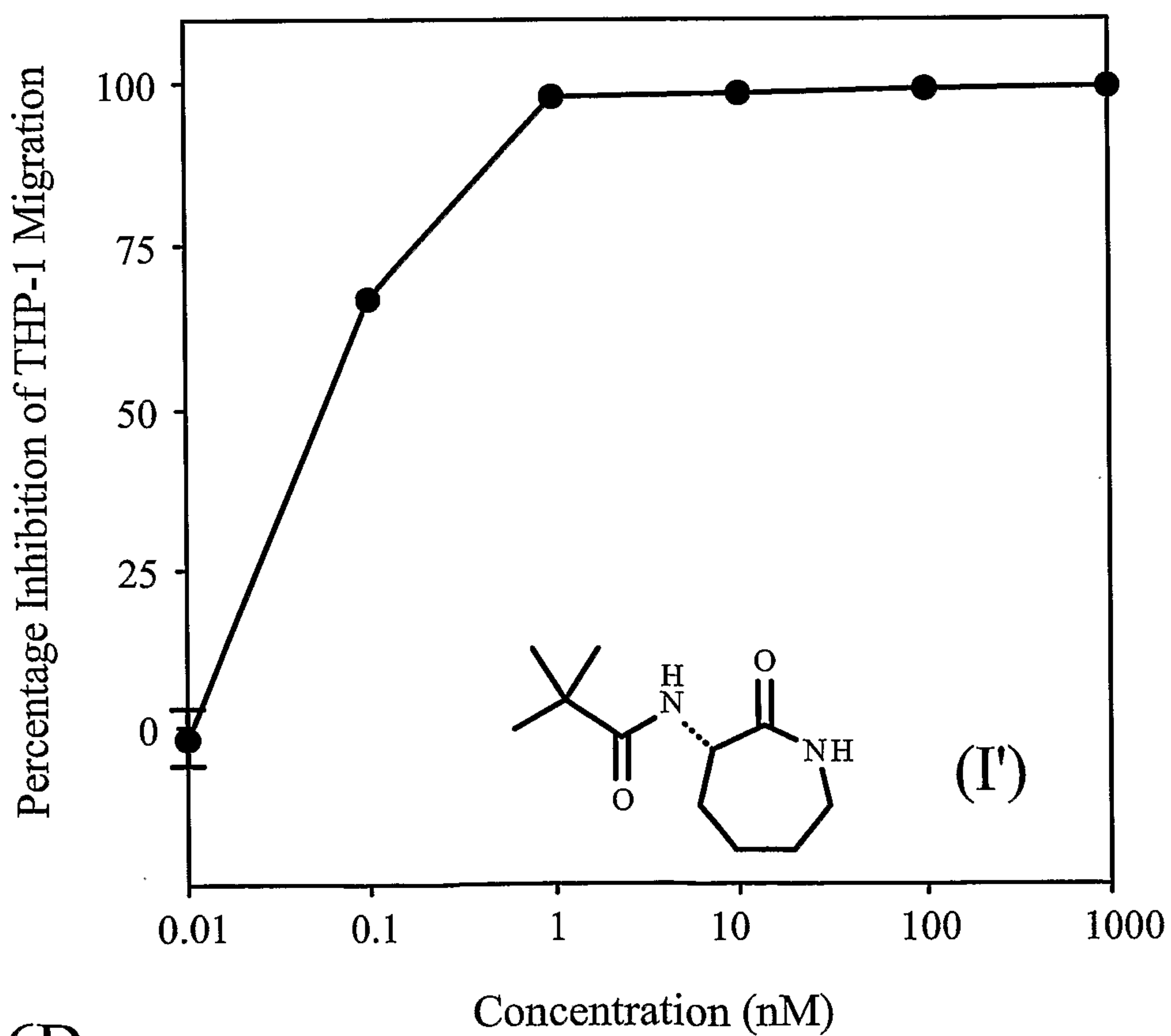
-13/21-

FIG. 5D

-14/21-

FIG. 5E

FIG. 6AFIG. 6B

FIG. 6CFIG. 6D

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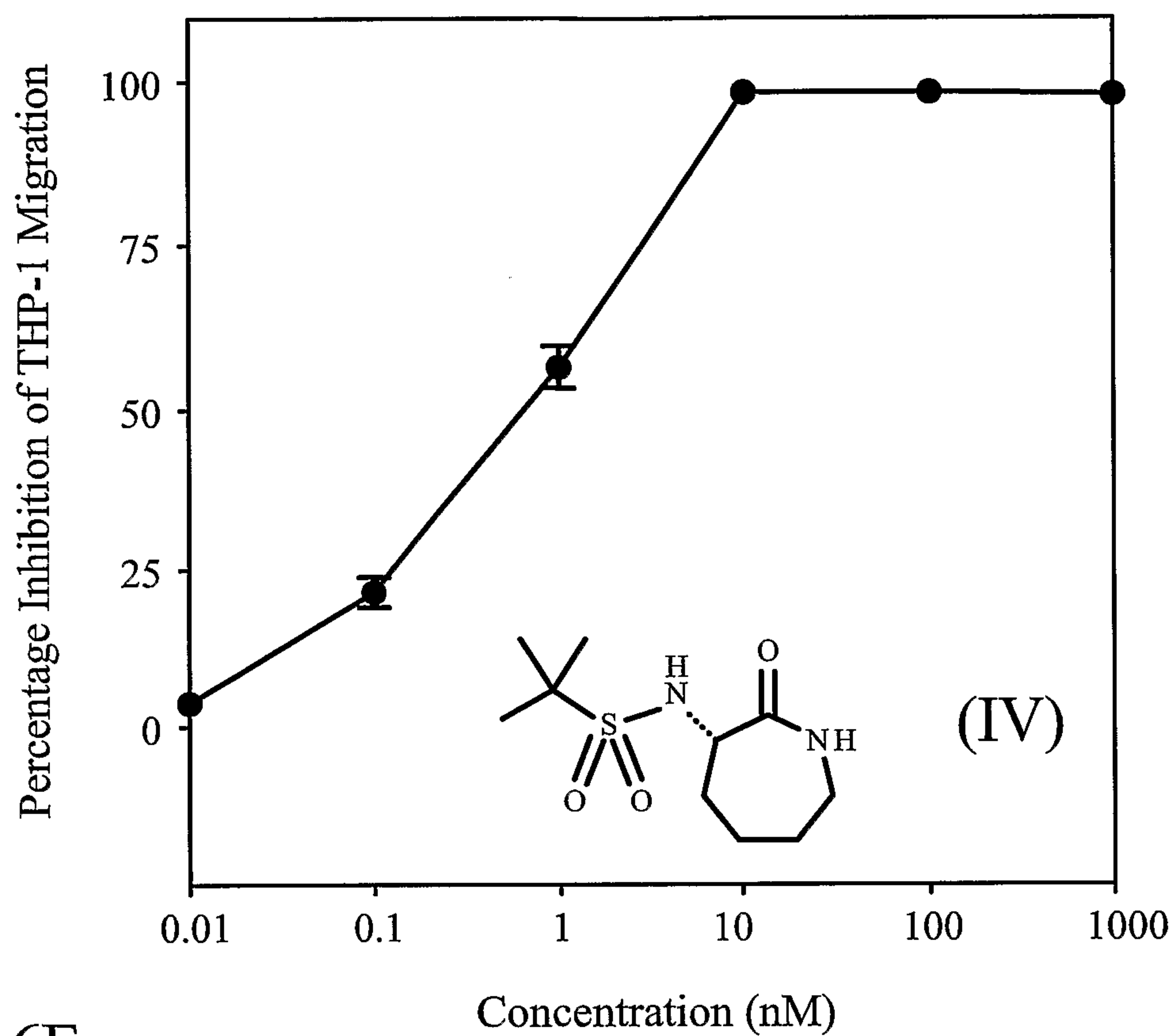


FIG. 6E

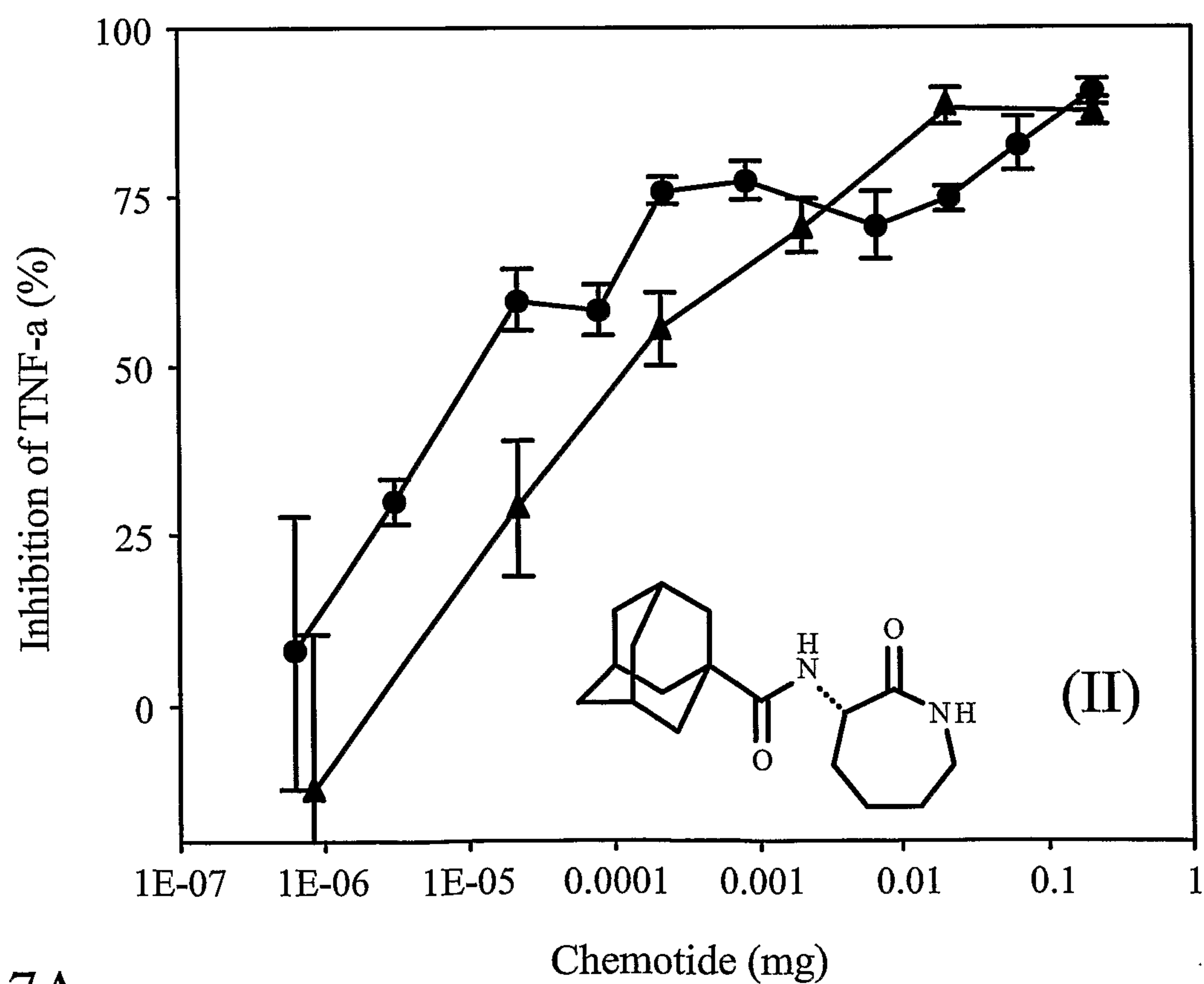
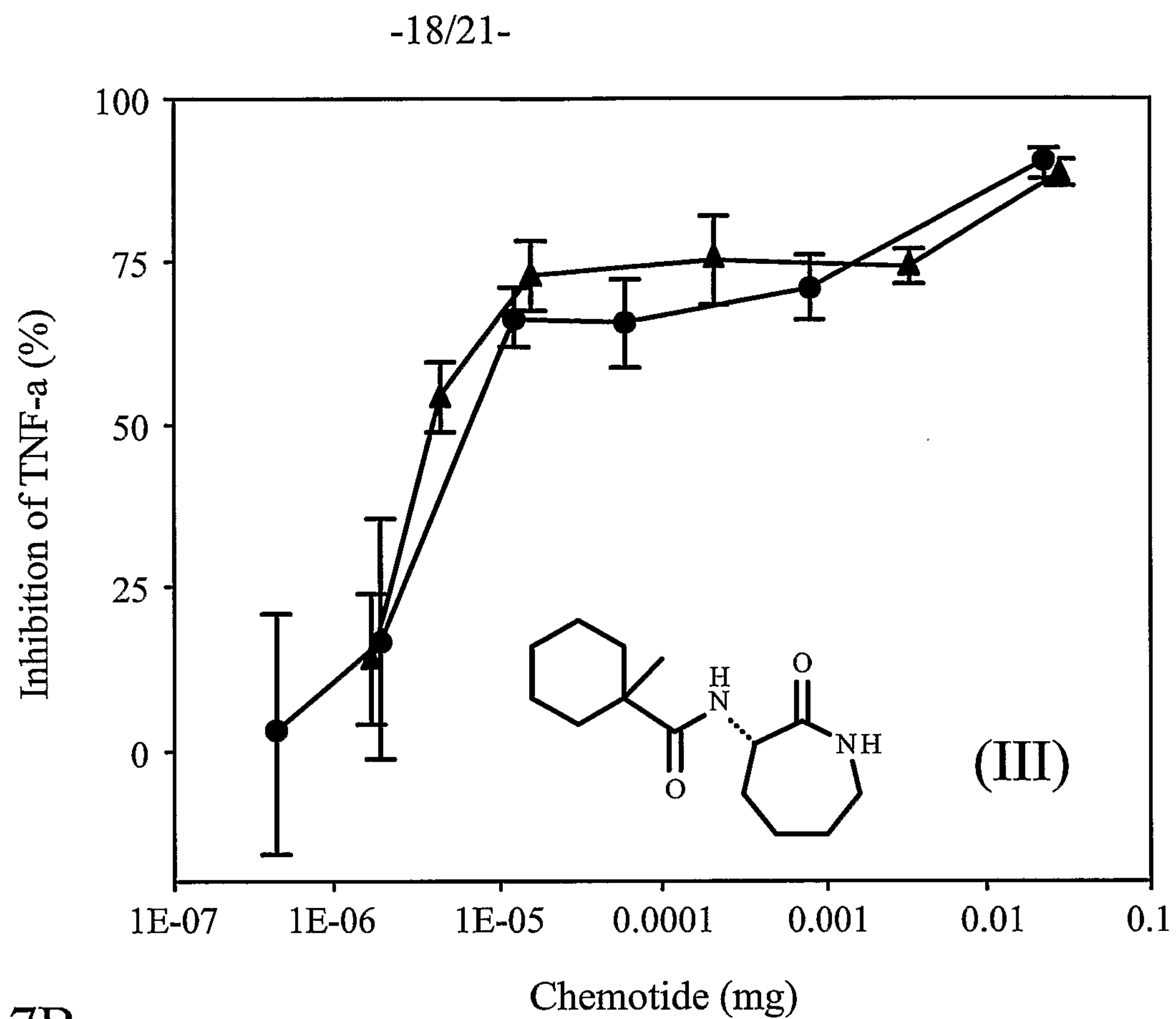
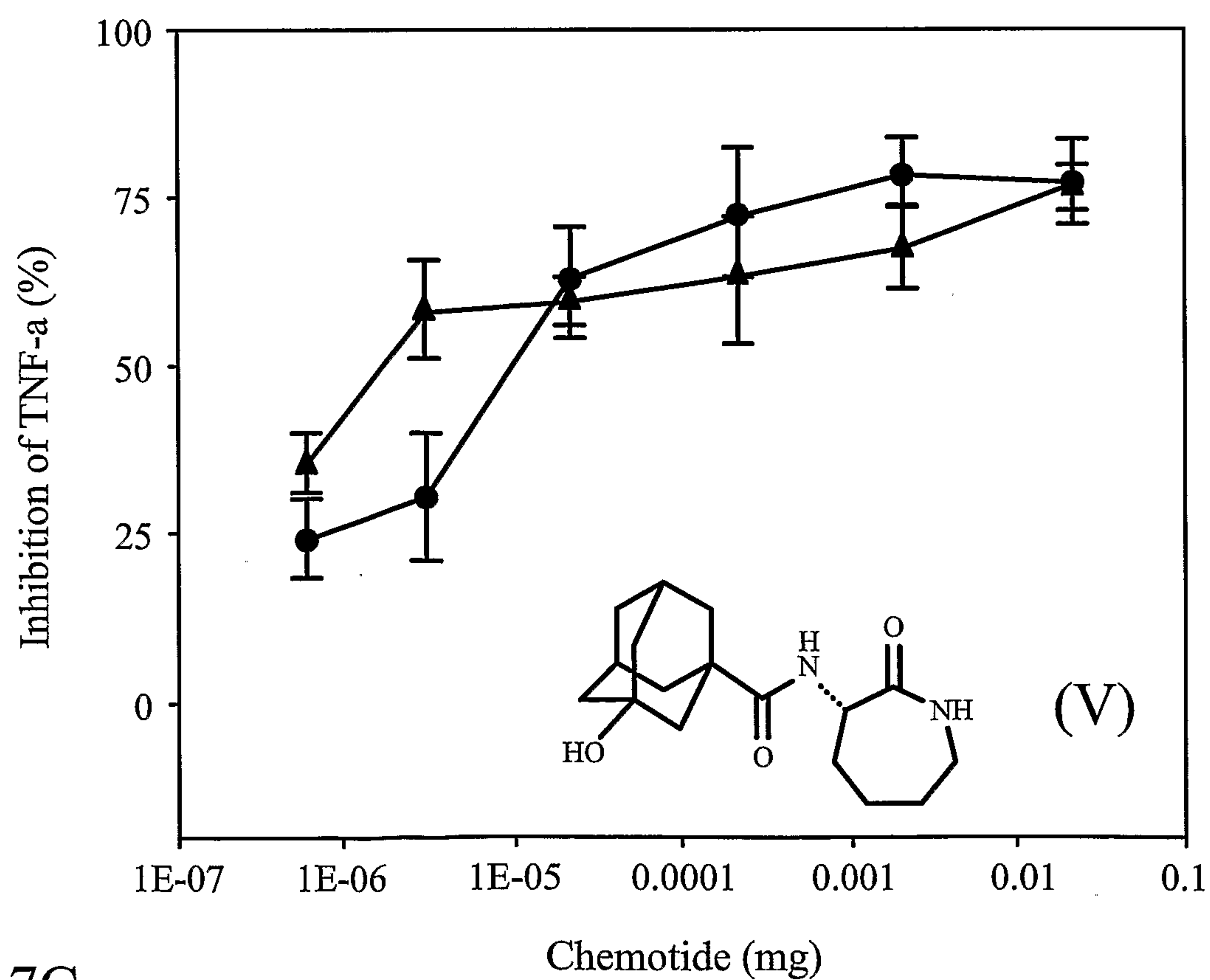
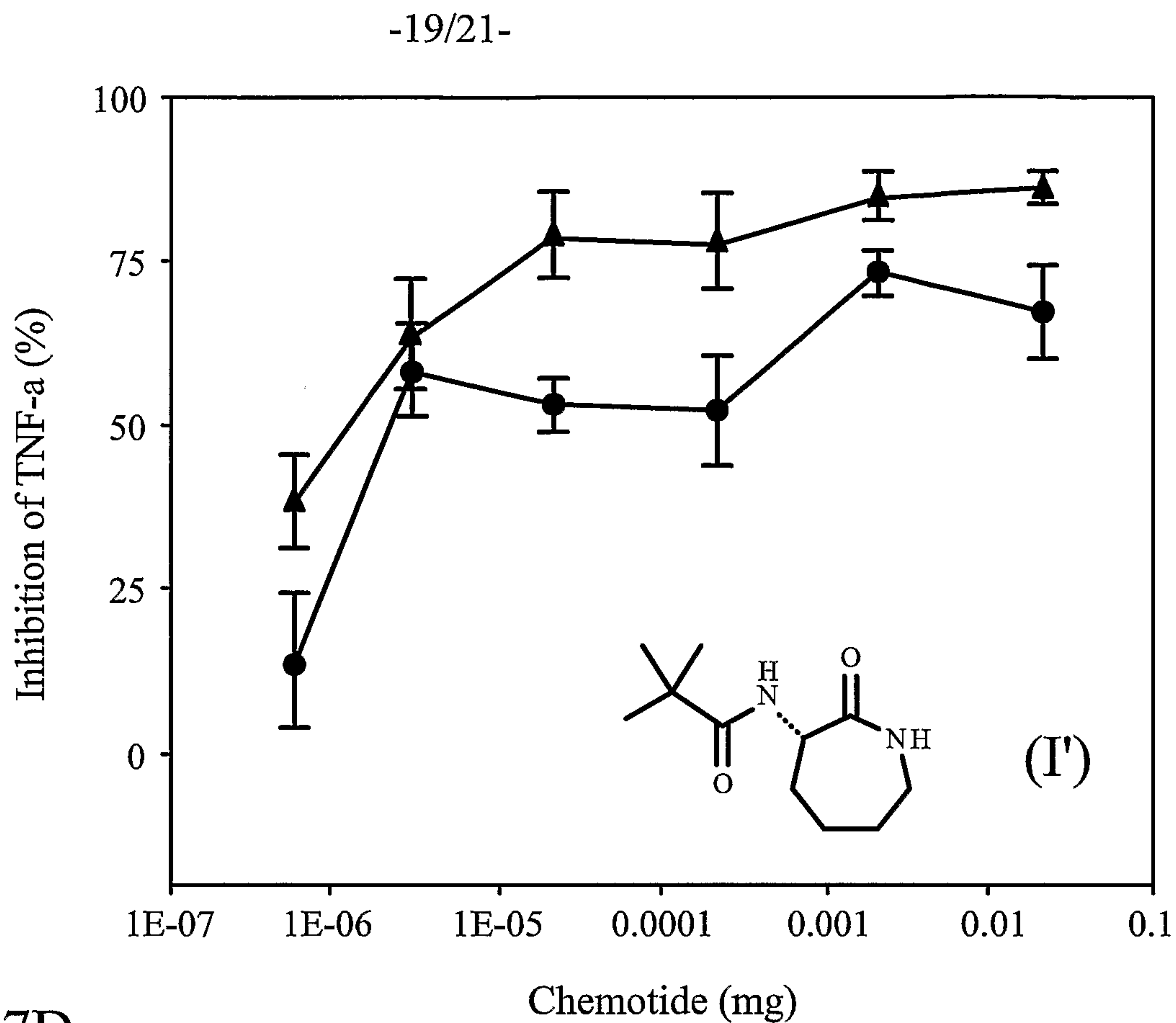
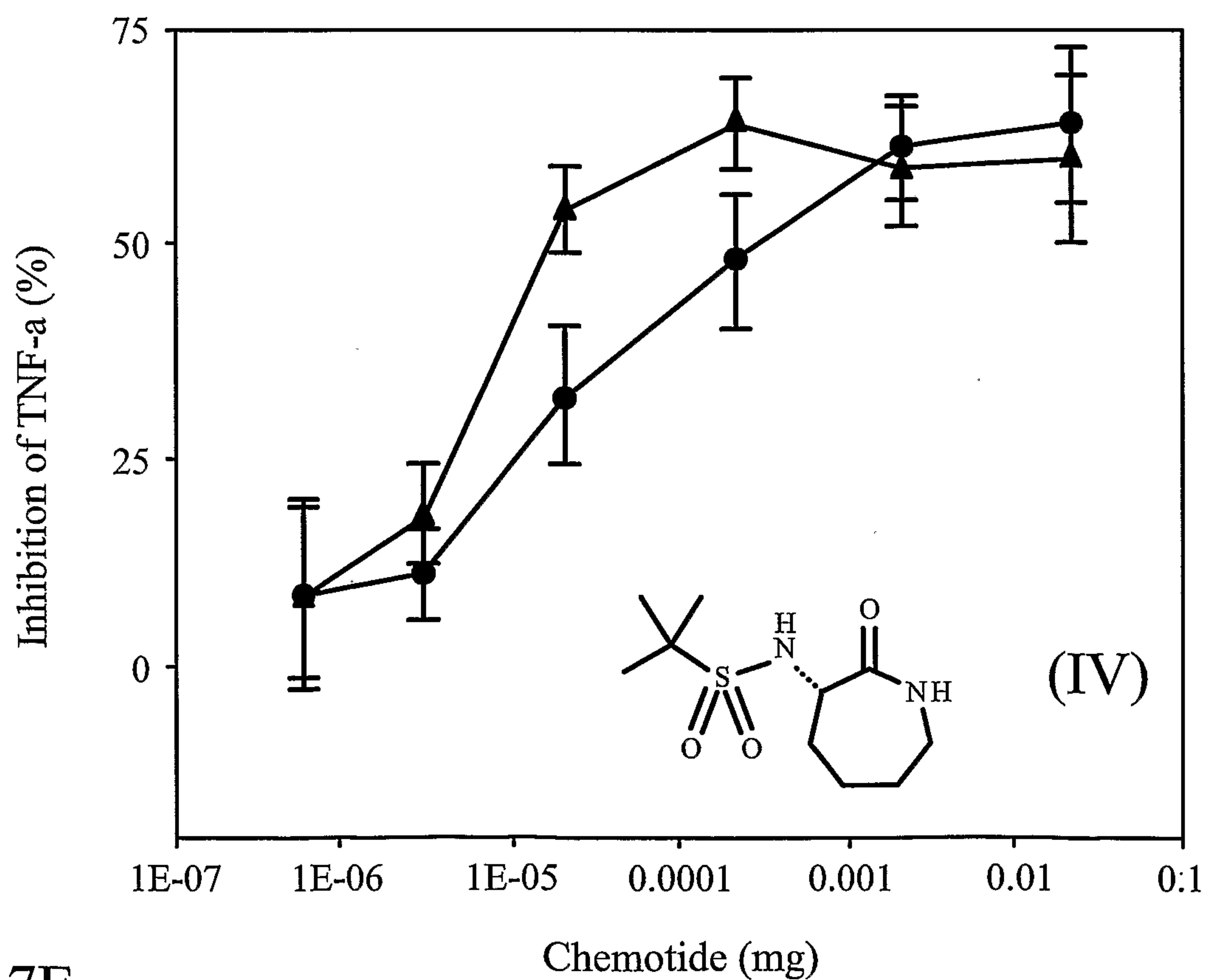
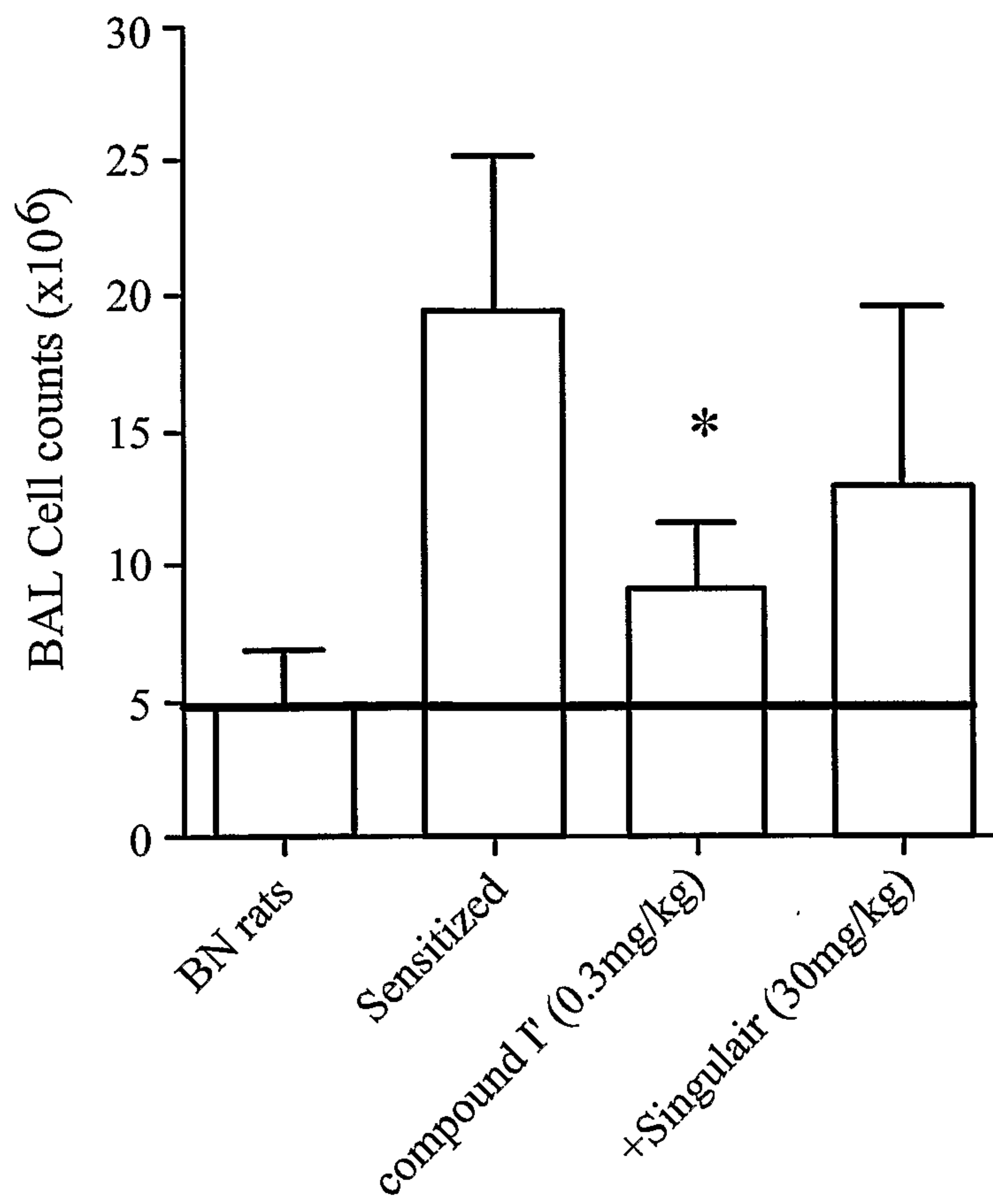
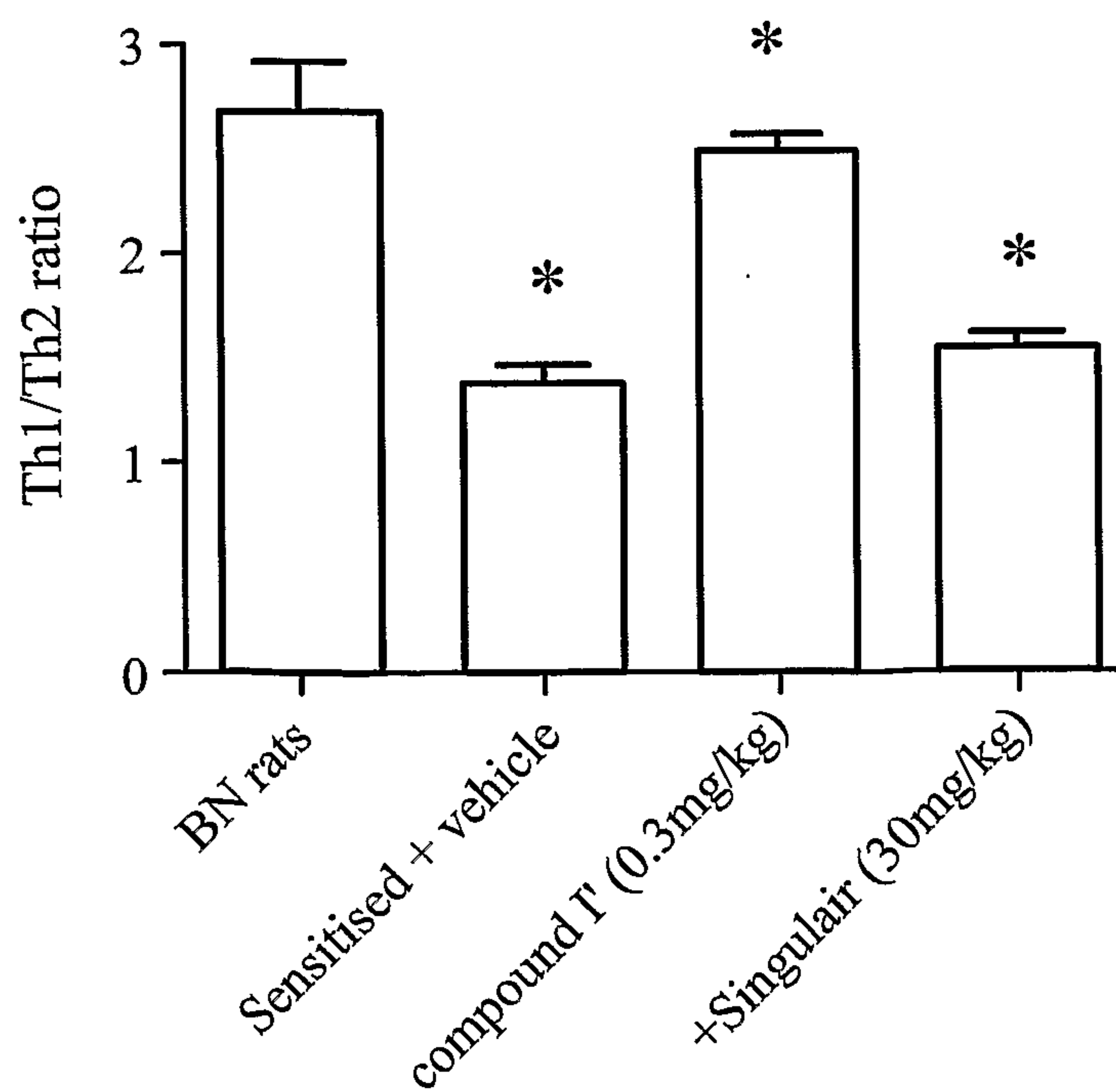


FIG. 7A

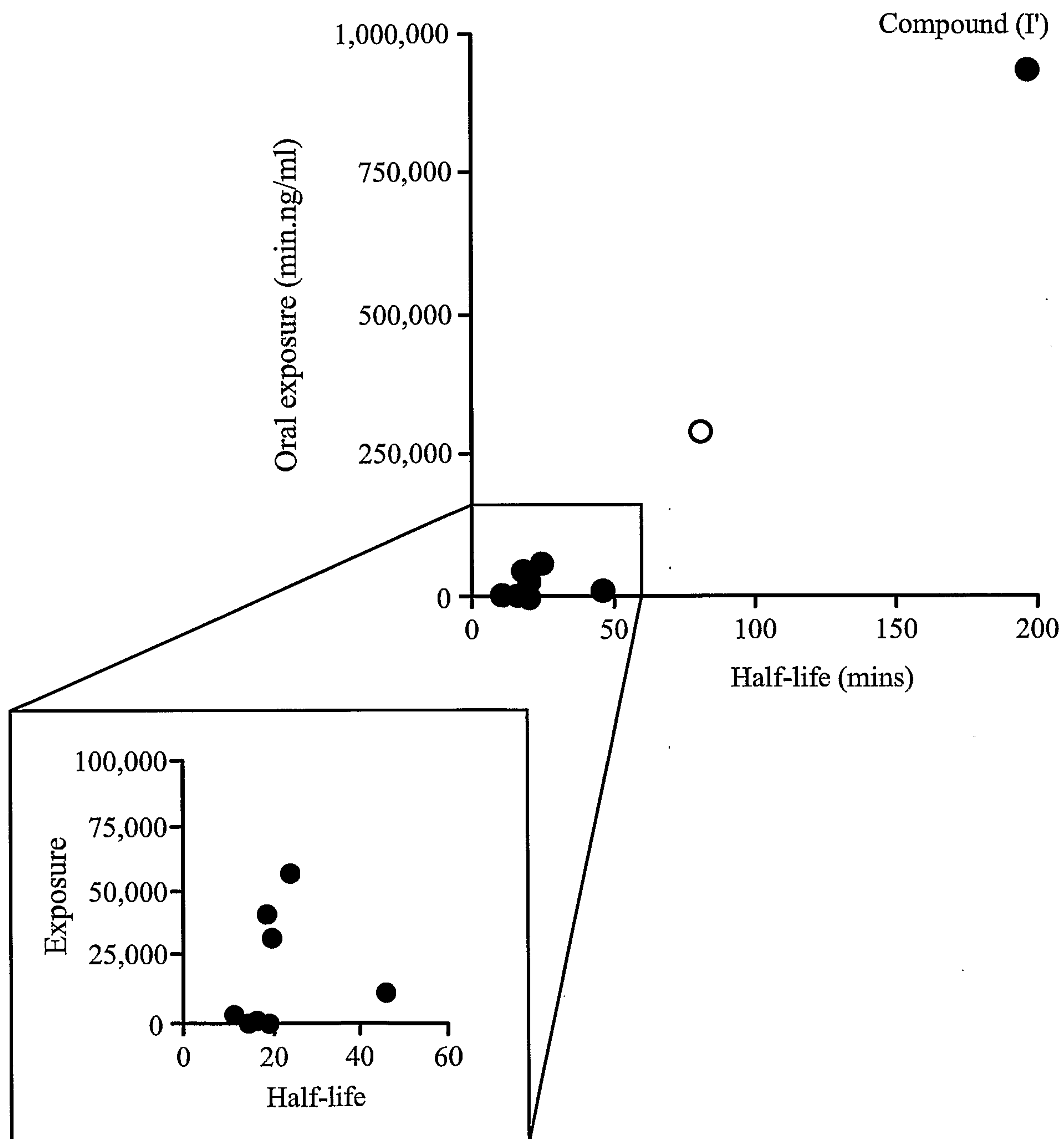
FIG. 7BFIG. 7C

FIG. 7DFIG. 7E

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FIG. 8FIG. 9

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FIG. 10