

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
25 June 2009 (25.06.2009)

PCT

(10) International Publication Number
WO 2009/078040 A2

(51) International Patent Classification:
C07C 229/26 (2006.01) A61K 31/198 (2006.01)
C07C 227/14 (2006.01)

(21) International Application Number:
PCT/IN2008/000836

(22) International Filing Date:
16 December 2008 (16.12.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1690/KOL/2007 17 December 2007 (17.12.2007) IN

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv))

Published:

— without international search report and to be republished upon receipt of that report

(54) Title: COMPOUNDS INDUCING REPERFUSION IN ISCHAEMIC TISSUES

(57) Abstract: This invention deals with a novel class of diamino alkanolic acids having a straight chain of seven to eleven carbon atoms with two terminal amino groups and a -COOH group (attached to alpha carbon atom) which are capable of effecting controlled formation of new blood vessels, and their pharmaceutically acceptable salts and/or derivatives thereof. The compounds may be in laevo, dextro, activated laevo, activated dextro or oligomeric form. The invention also pertains to a process for preparing the aforesaid novel class of compounds, which is illustrated by the accompanying drawing.



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TITLE OF THE INVENTION**COMPOUNDS INDUCING REPERFUSION IN ISCHAEMIC TISSUES**

The present invention relates to novel diamino C₇ – C₁₁ compounds having a straight chain with two terminal amino groups and a –COOH group, wherein the number of carbon atoms in the said side chain (R-group) varies between seven and eleven, said compounds being capable of effecting formation of new blood vessels in ischaemic tissues. More particularly this invention pertains to preparation and effective utilization of compound having a straight chain structure with number of carbon atoms in the varying between 7 and 11, additionally having a plurality of amino groups on the carbon atoms at two extremities and carrying a carboxyl group on α-carbon atom beside an amino group, said amino groups being capable of forming electrostatic and/or hydrogen bonds under condition of ischaemia following administration of said compounds resulting in controlled angiogenesis.

Ischaemic tissues are the organs or parts of human body which on occasions or under special circumstances get no or less supply of blood, either temporarily or permanently, due to spasm, obstruction or narrowing of blood vessels. Such incidence of deprivation of blood supply could be due to formation of blood clots or thrombi obstructing the blood vessel, or else the reason could be metabolic. Either way, the process of obstruction is gradual, spanning over years, though the symptoms of obstructions are discernible and results could be devastating, leading even to mortality.

The most common instance of ischaemic tissue leading to severe discomfort or/and death is ischaemic cardiac tissue or myocardium. The process of obstruction of the coronary vessel(s) is more often metabolic in nature, known as “atherosclerosis”, where a particular type of lipids get deposited on the interior walls of coronary vessels restricting or constricting the effective diameter over the years. During this process of vessel-lumen narrowing, the affected part of myocardium gets progressively decreasing amounts of requisite gases and nutrients. When the narrowing process

crosses a critical limit, the affected portion of myocardium dies in an acute circumstance known as 'myocardial infarction'.

A similar instance affecting increasing number of people is cerebral stroke where cerebral/neural tissues get affected due to lack of blood supply to the affected region.

Although the conditions may sound or appear to be different or divergent from each other, they do have a common denominator, namely, ischaemia or lack of blood supply to the tissues of the affected part. A common answer to both types of afflictions narrated above appears to be the process of revascularization, where either the available vessels are recanalized by removal of plaque formed therein, bypassed or grafted (replaced). A more prudent and safe mode of treatment would be development of collateral vessels supplying the ischaemic tissues.

Present day mode of treating cerebral stroke resides in the tPA therapy followed by physiotherapy. Now-a-days it is suggested that the therapy should be initiated within the first one and a half hour post stroke episode. Obviously such initiation of therapy is virtually impossible under Indian conditions, particularly in the far flung rural areas, not to speak of the cost involvement. Such therapy is affordable and accessible to only a handful few living in metropolitan cities having improved healthcare facilities. Moreover such tPA therapy has recently been known to exhibit an undesirable side effect, namely, induced break through bleeding in about 15 to 20% cases after administration.

As pointed out earlier, development of vessels supplying the ischaemic tissues known as "angiogenesis" or "vasculogenesis" is likely to address a large number of clinical conditions. The present invention deals with low-molecular weight, straight chain compounds with 7-11 membered carbon chain (R-Group) having amino groups at two terminal carbon atoms and a COOH group, having the capacity to form charged centres on N-atoms, which in turn bond with the charges residing on the receptor protein and angiogenic factor and/or growth factor. It has been observed that there is an

abundance of H^+ -ions in and around ischaemic tissues, in comparison with non-ischaemic tissues and these cause protonation of terminal amino groups of the compound, forming an ionized NH_3^+ -centre at the two ends, which in turn form electrostatic bonds with the negative charges residing both on the receptor protein (RP) present in the endothelial cell's membrane on the one hand and angiogenic factors on the other. The latter, namely, angiogenic factor or growth factor (AF/GF) acts as a ligand, establishing a link with the receptor by utilizing the charged terminals of the subject compound.

The angiogenic factors referred to above are known to be liberated in excess by the endothelial cells in the ischaemic tissue. These factors (AFs) have their receptors (a kind of protein) in the endothelial cell membrane. Establishment of a bridge by the protonated compound moiety brings about an activation of capillary with consequent angiogenesis through endothelial cell division and migration.

Compounds of this invention, namely, $C_7 - C_{11}$ diamino alkanoic acids may individually be represented as under:

- (i) 1,7-diamino-heptanoic acid
- (ii) 1,8-diamino-octanoic acid
- (iii) 1,9-diamino-nonanoic acid
- (iv) 1,10-diamino-decanoic acid, and
- (v) 1,11-diamino-ekadecanoic acid

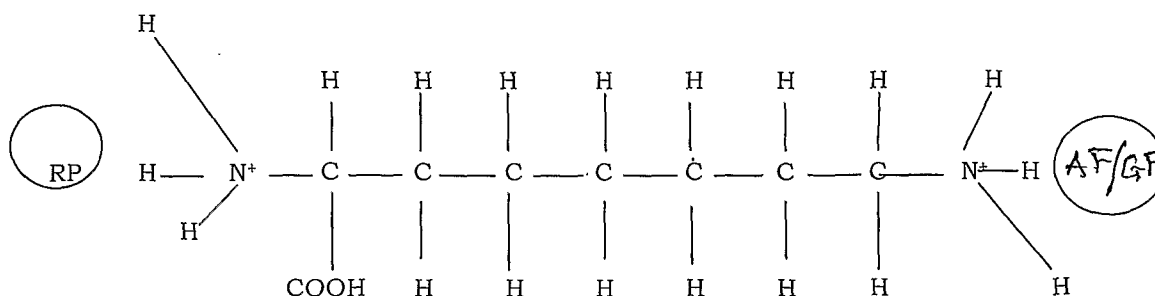
The foregoing compounds, either alone, or in various combinations, help in induction of new capillary formation in ischaemic tissue reperfusion in following instances, for example (non-exhaustive):

- (a) ischaemic cerebral/neural tissue(s), and as a preventive vaccine;
- (b) ischaemic myocardium/cardiac tissue(s) and as a preventive vaccine;

- (c) in ischaemic diabetic vasculopathy as in diabetic microangiopathy as in diabetic bone marrow, diabetic microangiopathy as in nephrons, diabetic wounds and ulcers and the like and as a preventive vaccine against development of diabetic microangiopathy;
- (d) in enhancing bioavailability of chemotherapeutic agent(s) and/or radio sensitizing agent(s) in case of ischaemic tumor tissue(s) during chemotherapy and/or radiotherapy, thereby enhancing efficacy of CT/RT, and
- (e) in induction of enhanced angiogenic response in reperfusion of various other ischaemic tissues like ischaemic renal tissue in ARF and CRF, ischaemic limb, ischaemic placental tissue, (as in IUGR) etc. and as a preventive agent against development of ischaemia in above tissues.

Preparation of the individual compounds may be done by following the scheme of reactions shown in the drawing accompanying this specification, which has been illustrated for the first representative member of the novel compounds of this invention, namely, 1,7-diamino-heptanoic acid. Adequate selection of the reaction component before arriving at intermediate nitrile compound (6) will result in higher homologues of diamino alkanoic acids with 8, 9, 10 or 11 number of carbon atoms in the chain. However, for the sake of convenience the experiments have been conducted with the C₇ compound as a representative member of the compounds with 7-11 carbon atoms in the carbon chain, each with a carboxyl group on the α -carbon atom and 2 terminal amino groups.

The activation of the 1,7-diamino-hexanoic acid, with consequent establishment of a bridge between RP (receptor protein) and AF/GF (angiogenic factor/growth factor) may be represented as follows:



The native representative compound as in (10) of Fig. I, gets activated by a process of protonation because of excess proton build-up in ischaemic tissues which triggers the change in conformation of the compound (e.g. diaminoheptanoic acid moiety), and enabling it to fit snugly between RP and AF/GF. Once a bridge is established, concerned cellular division and differentiation is augmented through a “more stable” receptor (RP) – ligand (AF/GF) complex on the endothelial cell membrane (and other cell membranes). This postulated “augmentation of receptor-ligand complex” is quite akin to enzyme-substrate interaction through the mediation of co-factors, and is not described in current biology and probably raises the possibility of a new concept in modern biology—“co-factor in receptor-ligand bridging/binding”. Similar considerations hold good for straight chain diamino alkanoic acids with 8, 9, 10 and 11 number of carbon atoms in the chain (R-Group). Formation of the aforesaid activated representative compound has been shown in Fig. 2 of the drawings.

The main object of this invention is to bring about angiogenesis in ischaemic tissues by employing a novel low molecular weight compound like 1,7-diamino-hexa-1-noic acid, a representative member selected from C₇-C₁₁ alkanoic acids having two terminal amino groups and a terminal –COOH group on α-carbon atom, pharmaceutically acceptable salts and/or derivatives thereof which are capable of bringing about formation of new blood vessels in ischaemic tissues. Such compound gets protonated and consequently activated in the ischaemic (e.g. in wound) bed thereby acting as a “micro-sensor”/“molecular sensor”, and the activated molecule bridges the AFs to their receptors, thereby accelerating the process of reperfusion of

the ischaemic (injured/ wounded) tissue bed. Such new low molecular weight compounds open up a completely new regime of wound/ischaemia management without any manifested side effects or contra-indications.

A further object of this is to provide novel C₇-C₁₁ alkanolic acids, having a straight chain with two terminal amino groups and a carboxyl group on the α -carbon atom.

A still further object of this invention is to provide a process for preparing the aforesaid novel compound(s) and their pharmaceutically acceptable salts and/or derivatives thereof, which may be in laevo, in dextro, active laevo, active dextro or oligomeric forms.

Another object of this invention is to provide a process for preparing the aforesaid novel compounds each with two terminal amino groups.

In accordance with this invention there is provided novel diamino alkanolic acids having a straight chain with two terminal amino groups and a -COOH group as described earlier, wherein the number of carbon atoms in the R-group in the side chain varies between 7 and 11, the said compounds being capable of effecting formation of new blood vessels (capillary) in ischaemic tissues, and/or pharmaceutically acceptable salts and/or derivatives thereof.

The present invention also relates to a process for preparing the aforementioned novel diamino alkanolic acids, and pharmaceutically acceptable salts and/or derivatives thereof, characterized in that the said preparation is carried out in accordance with the sequence of reactions shown in the accompanying drawing wherein the undernoted abbreviations have been used:

Cbz-Cl: Benzyloxy carbonyl chloride

THF: Tetrahydrofuran

PTSA: *p*-Toluene sulfonic acid

BH₃ DMS: Borane dimethylsulfide

PCC: Pyridinium chlorochromate

DCM: Dichloromethane and

(CH₂O)_n: Para formaldehyde

The following examples are given below based on the tests conducted, which are given by way of illustration and not by way of limitation.

Example – 1

Studies on the animal model

to ascertain the toxicity of the novel compounds

i. Animal model:

All animal experiments were performed following 'Principles of laboratory animal care' (NIH publication No.85-23, revised in 1985) as well as specific Indian laws on 'Protection of Animals' under the provision of authorized investigators. Swiss albino mice (~20g each; 10 – 12 weeks old; 3 mice in each group) were randomly divided into (i) untreated set (i.v. injected with PBS as vehicle only) and (ii) drug-treated set (Compound of this invention 0.1, 0.3, 1.0, 3.0, 10 and 30 mg/kg body weight) for 14 days. Untreated mice received sterile PBS as carrier vehicle. Doxorubicin (5 mg/kg body weight) was used as positive control for toxicity assays.

ii. Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) was conducted, followed by the Newman-Keuls multiple comparison test. Mean differences with $p < 0.05$ were considered statistically significant.

iii. Systemic toxicity

Hepato and cardio toxicity due to drug regimens are the shortfall in many instances. The serum levels of GOT, GPT and ALP are clinical indicators of

drug-induced toxicity. Blood samples were collected from the retro-orbital plexus of normal and the subject compound-treated mice. Total serum glutamate oxaloacetate transaminase (SGPT), glutamate pyruvate transaminase (SGPT), and alkaline phosphate were assayed according to standard protocols. In the aforesaid mice model, the levels of serum GOT and GPT were significantly increased in doxorubicin-treated mice ($p < 0.05$) which served as a positive control for toxicity assays. Doxorubicin treatment increased SGPT to 62 IU/dL, SGOT to 81 IU/dL and ALP to 45 KA units in 1,7-diamino alkanoic acid compound. Compound of this invention at doses between 0.1 mg/kg body weight and 30 mg/kg body weight showed no significant toxicity.

The data collected agreed well with the three independent sets of experiments done individually.

Example – 2

i. Screening of different cells

To study the toxic effects of compound (representative 1,7 diamino), a wide spectrum of primary cells or cell lines from various origin were screened for viability, using Trypan blue-exclusion test. Cells were grown in cultures in DMEM/F-12 (1:1) or RPMI 1640 media supplemented with 10% FBS, insulin (0.1 units/ml), L-glutamine (2 mM), sodium pyruvate (100 µg/ml), non-essential amino acids (100 µM), penicillin (100 units/ml) and streptomycin (100 µg/µl). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. Data collected showed that compound at doses 12.5 to 200 µg/mL range, showed no toxic effects (as the percent of cell killing was less than 5% upto 50 µg/mL doses which is 50 times more than the effective dose). In 100 and 200 µg/mL doses the drug showed mild toxicity which is even below the significant killing.

ii. **Effect of the novel compounds on in vitro toxicity on human cell lines**

Different cells from different origins were cultured *in vitro*. Various doses of the compounds of this invention were added in culture medium. After 24 h number of surviving cells was counted. Each experiment was performed in triplicate and the values agreed well with those of independent experiments as described in (i) of Example 2 above.

Example – 3

To test angiogenic potential of the compound

The angiogenic potential of the representative 1,7-diamino compound was tested by binding human VEGF to its putative receptors. Human lung epithelial cells (A549 which are highly metastatic and elaborates excess levels of angiogenic factor(s)) were grown on coverslip in DMEM/F-12 (1:1) or RPMI 1640 media supplemented with 10% FBS, insulin (0.1 units/ml), L-glutamine (2 mM), sodium pyruvate (100 µg/ml), non-essential amino acids (100 µM), penicillin (100 units/ml) and streptomycin (100 µg/µl). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The pH of the medium was maintained at 6.2 for the binding assay of VEGF to its receptor in the presence of compound. The cells were pre-treated with media alone or with 25 µg/ml compound for 30 min. The cells were then incubated with 10 ng/ml human VEGF for further 30 min at 37°C in a humidified atmosphere of 5% CO₂. The cells were fixed and stained with anti-VEGF antibody coupled with Alexaflour 488. The cells were visualized in Zeiss-confocal microscope.

Results

The results showed that normal A549 cell has low VEGF binding on the membrane, which remains unaltered after 1,7-diamino compound

treatment. Interestingly when cells were pre-incubated with said compound, the binding of VEGF to its receptor increased 10-15 times or even more.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without deviating or departing from the spirit and scope of the invention. Thus the disclosure contained herein includes within its ambit the various equivalents and substitutes as well.

Having described the invention in detail with particular reference to the illustrative examples given above and also to the accompanying drawing, it will be more specifically defined by claims appended hereafter.

I claim:

1. Novel diamino alkanolic acids having a straight chain with two terminal amino groups and a -COOH group on the α -carbon atom as shown in Fig. 2 of the accompanying drawings, wherein the number of carbon atoms in the said chain varies between 7 and 11, the said compounds being capable of effecting formation of new blood vessels in ischaemic tissues and pharmaceutically acceptable salts and/or derivative thereof.
2. Compounds as claimed in Claim 1, which are in laevo, dextro, activated laevo, activated dextro or oligomeric forms.
3. Compounds as claimed in Claims 1 and 2, either alone or in various combination(s) which help in induction of new capillary formation in ischaemic tissue reperfusion in the following instances, for example (non-exhaustive):
 - a. ischaemic cerebral/neural tissue(s) and as a vaccine for prevention of development of ischaemia in neural/cerebral tissue;
 - b. ischaemic myocardium/cardiac tissue and as a vaccine for prevention of development of myocardial ischaemia (angina);
 - c. in ischaemic diabetic vasculopathy as in diabetic microangiopathy in diabetic bone marrow, diabetic microangiopathy in nephrons, diabetic wounds and ulcers, and the like and as an agent for prevention of development of diabetic microangiopathy in any anatomic locations;

- d. in enhancing bioavailability of chemotherapeutic agent(s) and/or radiosensitizing agent(s) in case of treatment of ischaemic malignant tumor tissue(s) through/during chemotherapy/radiotherapy, thereby enhancing efficacy of CT/RT, and
- e. in inducing enhanced angiogenic response in reperfusion of various other ischaemic tissues like ischaemic renal tissue in ARF and CRF, ischaemic limb, ischaemic placental tissue (as in IUGR) etc., and as an agent for prevention of ischaemia in the above tissues as a vaccine.

4. A process for preparing the said novel diamino aknanoic having a straight chain with 7-11 carbon atoms and two terminal amino groups and a -COOH group on the α -carbon atom, and pharmaceutically acceptable salts and/or derivatives thereof as claimed in Claims 1 and 2, characterized in that the said compounds are prepared in accordance with the sequence of reactions shown in the accompanying drawing wherein the following abbreviations have been used:-

Cbz-Cl: Benzyloxy carbonyl chloride

THF: Tetrahydrofuran

PTSA: *p*-Toluene sulfonic acid

BH₃DMS: Borane dimethylsulfide

PCC: Pyridinium chlorochromate

DCM: Dichloromethane and

(CH₂O)_n: Para formaldehyde

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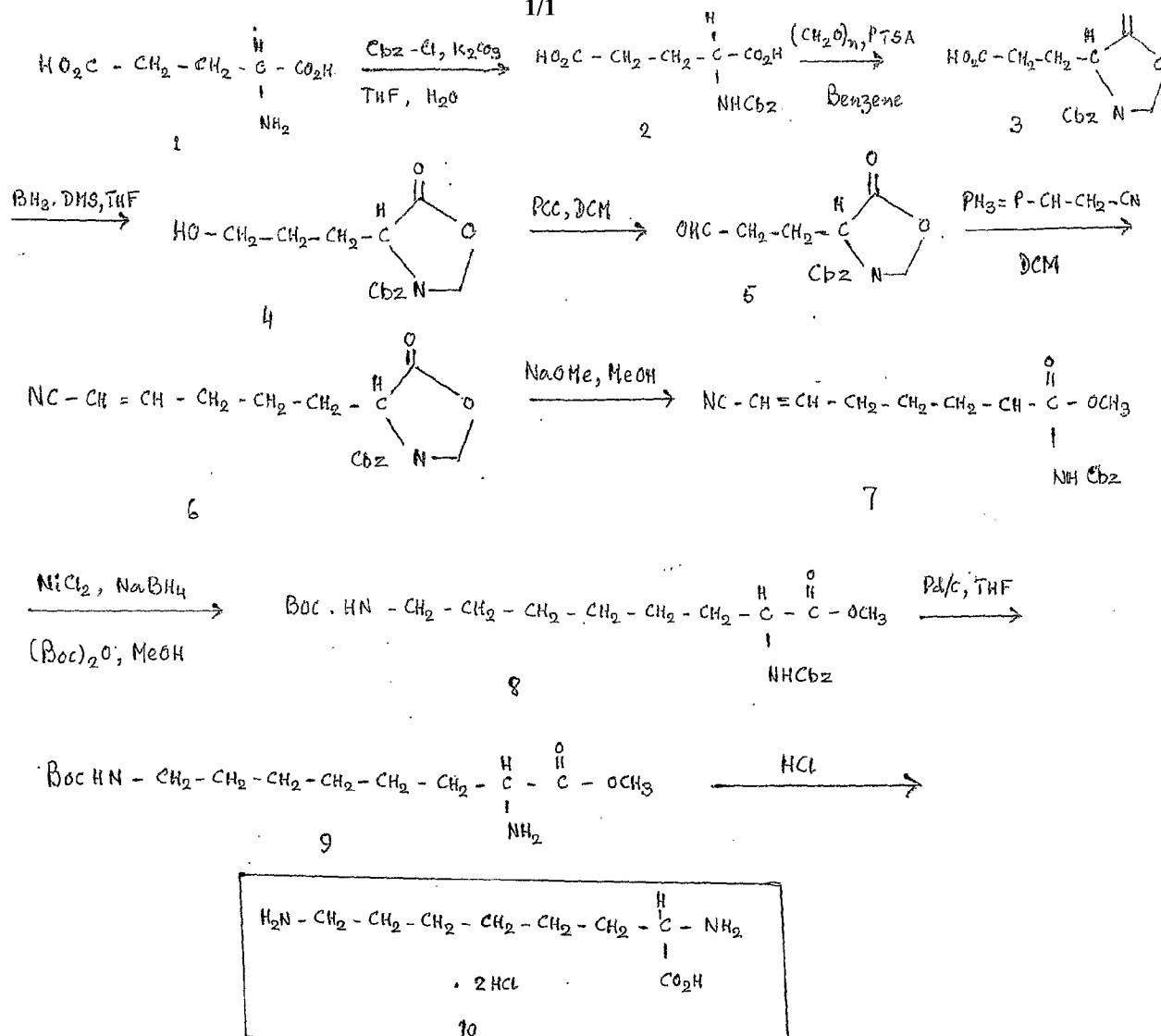


Fig. 1

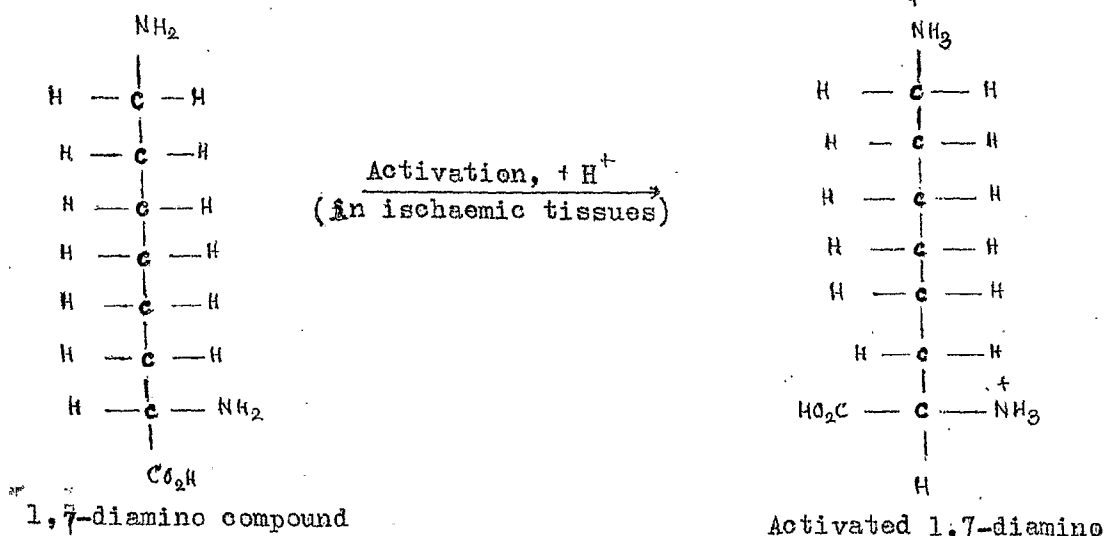


Fig. 2