Summary

Three steroid saponins from japanese Dioscoreaceae plants, dioscin, gracillin and kikuba-saponin were proved to have branched-chain oligosaccharide moieties, and assigned structures, diosgenin bis-α-L-rhamnopyranosyl(1→2 and 1→4)-β-D-glucopyranoside (IV), diosgenin α-L-rhamnopyranosyl(1→2_{Glc}.1)-β-D-glucopyranosyl(1→3_{Glc}.1)-β-D-glucopyranoside (V) and diosgenin β-D-glucopyranosyl(1→X_{Glc}.2)-β-D-glucopyranosyl(1→3_{Glc}.1)-α-L-rhamnopyranosyl(1→2_{Glc}.1)-β-D-glucopyranoside (VI), respectively.

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UDC 615.782.54-092.21

114. Satoshi Toki, Keiko Toki, and Hisao Tsukamoto : Metabolism of Drugs. XXXI.[1] Metabolic Fate of Methylhexabital (5-Cyclohexenyl-3,5-dimethylbarbituric Acid). (10).[2] Enzymatic Oxidation of Methylhexabital.

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In the enzymatic study of methylhexabital (MHB, 5-cyclohexenyl-3,5-dimethylbarbituric acid), Cooper and Brodie[3] found out that the enzyme in the rabbit liver which catalyzed an oxidation of the cyclohexenyl ring to yield keto-MHB was located in the microsomes of liver cells and required both oxygene and TPNH. Under a similar experimental condition, Tochino[4] observed that hydroxy-MHB was produced in the rabbit liver. Very regrettable, however, was the fact that in both cases unchanged MHB was extracted and assayed by the method of Brodie, *et al.*[5] and the enzyme activity was determined only with the value of disappeared MHB without regard to the metabolites themselves. In spite of the fact that while MHB disappeared, its metabolite are produced, as shown below, Cooper and Brodie[3] discussed only about the enzymatic mechanism of formation of keto-MHB, or only about the hydroxylation of MHB by Tochino.[4]

Previous studies in this laboratory showed that two diastereoisomeric α- and β-3-OH-MHB (α- and β-5-(3-hydroxy-1-cyclohexenyl)-3,5-dimethylbarbituric acid), 3-keto-MHB(5-(3-oxo-1-cyclohexenyl)-3,5-dimethylbarbituric acid) and three additional metabolites were excreted in the urine of rabbits administered MHB,[6,7] and that 3-OH-MHB and 3-keto-MHB were interconvertible *in vivo.*[2] Furthermore, it was confirmed that 3-OH-MHB and 3-keto-MHB were hardly separable on the paper chromatogram by the solvent system which those workers adopted since both compounds gave similar Rf

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1) Part XXX : M. Yoshimura, H. Tsukamoto : This Bulletion, (in press).
2) H. Tsukamoto, S. Toki, K. Kaneda : *Ibid.*, **7**, 651 (1959).
3) J.R. Cooper, B.B. Brodie : J. Pharmacol. Exptl. Therap., **114**, 409 (1955).
4) Y. Tochino : Wakayama Med. Repts., **4**, 119 (1958).
5) B.B. Brodie, J.J. Burns, L.C. Mark, P.A. Lief, E. Bernstein, E.M. Papper : J. Pharmacol. Exptl. Therap., **109**, 26 (1953).
6) H. Tsukamoto, H. Yoshimura, S. Toki : This Bulletin, **4**, 368 (1956).
7) H. Yoshimura : *Ibid.*, **5**, 561 (1957).

values.[8] However, these compounds and other metabolites could be nicely separated by using buffered paper chromatography which was devised in this laboratory,[8] and the method of simultaneous determination of main metabolites of MHB by buffered paper chromatography in conjunction with ultraviolet spectrophotometry were also established.[9]

The present study has been undertaken in order to clarify more exactly the MHB oxidation mechanism and metabolic pathway *in vitro* employing the above mentioned method. This paper describes that MHB is metabolized at first to 3-OH-MHB by a TPNH*[2]-dependent enzyme in the liver microsomes, and then to 3-keto-MHB by an enzyme system localized in the soluble fraction of the liver. In this case, enzyme activity was determined by the value of the respective metabolites, 3-OH-MHB and 3-keto-MHB.

A part of this report has already been presented in the preliminary communication.[10]

Materials and Methods*[3]

Materials——MHB (m.p. 142~143°) was supplied by the Dainippon Pharmaceutical Co. Ltd. 3-Keto-MHB (m.p. 160~161°) was prepared by the CrO_3-oxidation of MHB.[11] 3-OH-MHB(α-form, m.p. 213~215°(decomp.); β-form, m.p. 141~142°(decomp.)) were prepared by the Meerwein-Ponndorf reduction of 3-keto-MHB.[12] TPN and DPN of approximately 100% purity were purchased from the Sigma Chemical Company. TPNH was prepared by the method of Kaplan, *et al.*[13] Glucose-6-phosphate dehydrogenase (type V) was purchased from the Sigma Chemical Company. Diethylaminoethyl diphenylpropylacetate HCl (SKF 525-A) was donated by Smith, Kline and French Laboratories.

Preparation and Fractionation of Liver Homogenates——Preparation of the liver fractions was carried out at 0 to 3°. Male albino rabbits (body weight, about 3 kg.) were killed by a blow on the head, and the livers were immediatly removed and homogenized in 2 volumes of 0.1*M* phosphate buffer (pH 7.4) (containing 30 m*M* nicotinamide and 4 m*M* $MgCl_2$) for 1.5 min. in a Potter-Elvehjem type Teflon homogenizer. The homogenate was centrifuged at 600×g for 10 min. to remove whole cells, nuclei and cellular debris, and the supernatant was centrifuged at 9,000×g for 20 min. to remove mitchondria. The 9,000×g supernatant containing microsomes and the soluble fraction was centrifuged at 80,730×g for 1 hr. in a Hitachi model 40 P ultracentrifuge. Microsomes were separated, washed with 0.1*M* phosphate buffer (pH 7.4) and recentrifuged. The washed nuclei, mitchondria and microsomes were resuspended in 0.1*M* phosphate buffer (pH 7.4) equivalent to the volume of the original liver.

Enzymatic Assay——MHB was incubated with the various preparations in a metabolic shaker for 1 hr. at 37° as indicated in each of the Tables. Then enzyme activity was determined by measuring the quantities of unchanged MHB and its oxidation products, 3-OH-MHB and 3-keto-MHB.

Analytical Method——After incubation the reaction mixture was transferred into a 50 cc. glass-stoppered centrifuge tube, heated for 2 min. on a boiling water bath, then 3 g. of NaCl, 0.5 cc. of 1*N* HCl and 25 cc. of AcOEt were added to the tube, and the mixture was shaken mechanically for 30 min. The tube was centrifuged and 25 cc. of the AcOEt layer was pipetted out followed by addition of 30 cc. of fresh AcOEt to the tube. The mixture was shaken for 15 min. and 30 cc. of the AcOEt layer was pipetted out after centrifugation. The combined AcOEt extracts were washed with 10 cc. of 0.1*N* HCl and 5 cc. of H_2O, treated with 0.3 g. of activated charcoal, filtered and the filtrate was evaporated to dryness. The residue was dissolved in 0.6 cc. of EtOH and 0.1 cc. of this solution was applied on a filter paper. Samples were subjected to paper chromatography for 17 hr. on Toyo Roshi No. 50 (2×40 cm.) which was previously treated with borate buffer (pH 10), by the

*2 Abbreviations used : TPN, triphosphopyridine nucleotide; TPNH, reduced TPN; DPN, diphosphopyridine nucleotide; FAD, flavine adenine dinucleotide.

*3 All melting points are uncorrected.

8) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, **6**, 15 (1958).
9) *Idem* : *Ibid.*, **6**, 88 (1958).
10) H. Tsukamoto, S. Toki, K. Toki : *Ibid.*, **8**, 561 (1960).
11) H. Tsukamoto, H. Yoshimura, S. Toki : *Ibid.*, **4**, 364 (1956).
12) H. Yoshimura : *Ibid.*, **6**, 13 (1958).
13) N.O. Kaplan, S.P. Colowick, E.F. Neufeld : J. Biol. Chem., **195**, 107 (1952).

ascending technique using BuOH saturated with the same buffer (1:1) as the solvent. After development the strips were removed and air-dried. The position of metabolites was detected by an ultraviolet lamp or by spraying with 1% $NaIO_4$ and 1% $KMnO_4$ solutions. The sections of unchanged MHB, 3-OH-MHB, *4 and 3-keto-MHB were cut out, eluted with 5.0 cc. or 10.0 cc. of borate

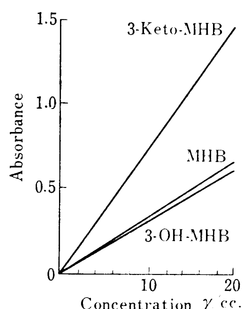


Fig. 1. Relation between Extinction of Absorption Peak and Concentration of MHB, 3-OH-MHB*5 or 3-keto-MHB*6

buffer (pH 10), and the concentration of metabolites was determined by measuring the absorbance of the absorption peaks at the respective wave lengths (MHB and 3-OH-MHB,*5 244 mμ; 3-keto-MHB,*6 239 mμ). For the blank, the same areas of buffered paper as the corresponding spots were examined in parallel. The blank value agree correspondingly with the values obtained for the extract from the reaction mixture which was free from MHB. Recovery rate was 82% for MHB, 76% for 3-OH-MHB and 73% for 3-keto-MHB.

Detection and Identification of Metabolites——The extracts from the reaction mixture were chromatographed as described above, and the position of each spot was compared with that of the authentic sample. Moreover, the compounds detected were confirmed by the ultraviolet spectra of the effluents from the paper.

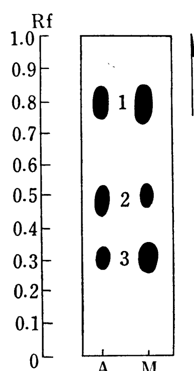


Fig. 2. Paper Chromatogram of MHB Metabolites

A : Authentic Samples
M : Metabolites
1 : MHB
2 : 3-OH-MHB*3
3 : 3-Keto-MHB

Results

Detection and Identification of MHB Metabolite——A mixture of 6 μmoles of MHB, 100 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, 4 cc. of rabbit liver homogenate made to 8 cc. with 0.1*M* phosphate buffer (pH 7.4) was incubated for 60 minutes at 37°. The extract of the incubation mixture was chromatographed on buffered paper, and the authentic samples of MHB, 3-OH-MHB and 3-keto-MHB were run in parallel. Three spots corresponding to unchanged MHB, 3-OH-MHB and 3-keto-MHB appered in the

*4 The two diastereoisomeric α- and β-3-OH-MHB have identical Rf values and cannot be distinguished by the chromatographic procedure used.

*5 α- and β-3-OH-MHB have identical molar extinction coefficients.

*6 Absorption of 3-keto-MHB was measured after 2 hr., since this compound was readily decomposed in this solution.

extract of the incubated MHB. These spots exhibited the same Rf values and absorption spectra as authentic samples (Fig. 2). Therefore, it was clear that MHB was metabolized to 3-OH-MHB and 3-keto-MHB with the liver homogenate.

Intracellular Localization of Enzyme Activity——The whole homogenate and the 600×g and 9000×g supernatant fractions of liver catalyzed the oxidation of MHB to 3-OH-MHB and 3-keto-MHB. Neither the soluble nor the microsomal fractions showed any activity, but the combination of these two fractions catalyzed also the formation of the same two kinds of metabolites as shown above (Table I).

These data indicated that the biotransformation of MHB to 3-OH-MHB and 3-keto-MHB required both microsomal and soluble fractions of liver.

Table I. Intracellular Localization of Enzyme Activity

Fractions	Unchanged MHB (%)	3-OH-MHB formed (%)	3-Keto-MHB formed (%)
Whole homogenate	10.5	37.4	38.9
Nuclei	100	0	0
600×g supernatant fraction	28.3	18.8	40
Mitchondria	97	0	0
9,000×g supernatant fraction	19.6	28.0	42.8
Microsomes	100	0	0
Soluble fraction	97.2	0	0
Microsomes+Soluble fraction	38.2	15.3	39.3

Condition : Incubation flasks contained 100 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, 4 cc. of tissue fraction (equivalent to 1.3 g. of the liver), 6 μmoles of MHB, and 0.1*M* phosphate buffer (pH 7.4). The total volume was 8.0 cc. and incubated for 1 hr. at 37°.

Requirements for Oxidation of MHB——When the 9000×g supernatant fraction was dialyzed for 21 hours at 0° against 0.1*M* phosphate buffer (pH 7.4) (containing 30 m*M* of nicotinamide and 4 m*M* of magnesium chloride), its activity was markedly decreased. However, the activity could be restored by the addition of TPN but not of DPN (Table II). Neither 3-OH-MHB nor 3-keto-MHB was formed under anaerobic conditions.

Hence, it was confirmed that both TPN and oxygen were required for the oxidation of MHB by the liver supernatant fractions.

Table II. Effect of DPN and TPN on the Metabolism of MHB in Dialyzed 9,000×g Supernatant Fraction

Components	Unchanged MHB (%)	3-OH-MHB formed (%)	3-Keto-MHB formed (%)
9,000×g supernatant fraction	12.9	25.1	44.2
Dialyzed 9,000×g supernatant fraction	77.5	1.6	3.2
Dialyzed 9,000×g supernatant fraction + DPN	71.9	3.7	8.5
Dialyzed 9,000×g supernatant fraction + TPN	29.7	16.7	42.8

Condition : Incubation flasks contained 100 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, 4 cc. of dialyzed 9,000×g supernatant fraction, 6 μmoles of MHB, 0.75 μmole of DPN or TPN, and 0.1*M* phosphate buffer (pH 7.4). The total volume was 8.0 cc. and incubated for 1 hr. at 37°.

Hydroxylation of MHB by Microsomal Enzyme System——As shown in Table III, when MHB was incubated with microsomes and the soluble fraction in which TPN participated as coenzyme, both 3-OH-MHB and 3-keto-MHB were formed. When MHB was incubated with microsomes and TPN only, a marked decrease of activity was observed. MHB was, however, metabolized by microsomes if it was incubated with TPNH or TPN and a TPNH generating system consisting of glucose-6-phosphate and

Table III. Requirement for TPNH in Hydroxylation of MHB by Liver Microsomes

Components	Unchanged MHB (%)	3-OH-MHB formed (%)	3-Keto-MHB formed (%)
Microsomes + Soluble fraction	23.4	22.2	41.0
Microsomes + TPN	100	1.8	0
Microsomes + TPNH[a]	78.0	22.6	4.1
Microsomes + TPN + Glucose 6-phosphate + Glucose-6-phosphate dehydrogenase	67.8	30	3.6

Condition : Incubation flasks contained 100 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, microsomes equivalent to 1.3 g. of liver, 6 μmoles of MHB, cofactors as indicated, and 0.1*M* phosphate buffer (pH 7.4). The total volume was 8.0 cc. and incubated for 1 hr. at 37°. 0.75 μmole of TPN, 30 μmoles of glucose-6-phosphate, and 10 units of glucose 6-phosphate dehydrogenase were added as indicated.

a) 1 μmole of TPNH was added at 10 min. intervals to the incubation system.

glucose 6-phosphate dehydrogenase. In the both experiments, MHB was converted to 3-OH-MHB and only a negligible amount of 3-keto-MHB was obtained.

From these data it was concluded that hydroxylation of MHB occurred only in the TPNH-dependent microsomal enzyme system and moreover, this first metabolite, 3-OH-MHB, was converted to 3-keto-MHB by an enzyme system located in the soluble fraction, since the amount of 3-keto-MHB was markedly increased when MHB was incubated with microsomes and the soluble fraction instead of TPNH.

Inhibitors of MHB Metabolism——The MHB hydroxylation system was inhibited by methylene blue, menadione, riboflavine, FAD, chlorpromazine, *p*-chloromercuribenzoate, ascorbic acid and *α,α'*-dipyridyl but potassium cyanide had no influence. SKF 525-A, a compound which inhibits the metabolic transformation of many drugs by microsomes, also exhibited inhibition (Table IV).

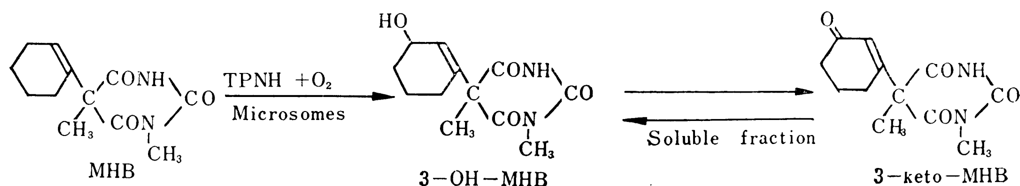
Table IV. Effect of Various Inhibitors on MHB Metabolism

Inhibitor	Concentration (*M*)	Inhibition (%)	Inhibitor	Concentration (*M*)	Inhibition (%)
Methylene blue	1×10^{-3}	98	Chlorpromazine hydrochloride	1×10^{-3}	68
Menadione	1×10^{-3}	95	*p*-Chloromercuribenzoate	1×10^{-3}	29
Riboflavine	1×10^{-4}	92	Ascorbic acid	1×10^{-3}	14
FAD	6.8×10^{-4}	85	*α,α'*-Dipyridyl	1×10^{-3}	12
SKF 525-A	1×10^{-3}	79	Potassium cyanide	1×10^{-3}	0

Condition : Incubation flasks contained 100 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, 4 cc. of 9,000×g supernatant fraction, 6 μmoles of MHB, inhibitors as indicated, and 0.1 *M* phosphate buffer (pH 7.4). The total volume was 8,0 cc. and incubated for 1 hr. at 37°.

Discussion

The experiments described in this paper have clearly indicated that MHB is hydroxylated to 3-OH-MHB and detoxicated by a TPNH-dependent enzyme in rabbit liver microsomes. As was reported in the previous communication from this laboratory, 3-OH-MHB and 3-keto-MHB were also interconvertible in the soluble fraction of liver.[10] The results obtained from these experiments demonstrated that MHB was metabolized along the oxidative pathway shown below :



These conclusion were drawn by employing the method of simultaneous determination of 3-OH-MHB, 3-keto-MHB and unchanged MHB.

Cooper and Brodie[3] showed that MHB was oxidized in rabbit liver homogenate to yield a single metabolite, keto-MHB, and that this oxidative system was located in the microsomes and required both TPNH and oxygen. Tochino[4] also examined the metabolic fate of MHB and reported that hydroxy-MHB but no keto-MHB was produced by homogenate and 9000×g supernatant fraction. These workers adopted the same technique in determining enzyme activity by measuring the unchanged MHB, but, they obtained respectively different oxidation products under similar experimental conditions and expressed different views on the oxidation mechanism and pathway of MHB. This contradiction in interpretation of the mechanism about metabolic pathway of MHB was eliminated by the present experiment.

The necessity of TPNH in a oxidative reaction is unusual, but similar type of reactions have been found already by Brodie, *et al.*,[14,15] who reported that metabolism of a number of drugs and foreign compounds is catalyzed by liver microsomes; these reactions include, for example, side chain oxidation of barbiturates, hydroxylation of aromatic rings, cleavage of ethers, deamination of chlorpromazine sulfoxide from chlorpromazine, etc.

According to the viewpoint that these reactions require TPNH and oxygen, this drug-metabolizing enzyme system might belong to mixed function oxygenase.[16] The mechanism of these oxidations is not yet clarified but some types are proposed by Mason.[17]

Various efforts to solubilize the microsomal drug-metabolizing enzyme were made by Cooper and Brodie[3] but unsuccessful. Attempts to obtain the solubilized microsomes which hydroxylate MHB are now in progress in the authors' laboratory. Recently, Imai and Sato[18,19] reported that a lipid-like component of rabbit liver microsomes was essential for the hydroxylation of aniline by the same granules, and that solubilized aniline hydroxylase could be obtained with the aid of heat-treated venom of *Trimeresurus flavoviridis*. At present, it is supposed that the microsomal enzyme catalyzes the nonspecific oxidation of drugs and foreign compounds, but this problem would be solved by investigating at least both aromatic and aliphatic hydroxylations using a solubilized and purified enzyme system.

On the side chain oxidation of *p*-nitrotoluene, Gillette[20] showed that *p*-nitrobenzyl alcohol was oxidized to *p*-nitrobenzaldehyde by a DPN-dependent enzyme in the soluble fraction of liver or alcohol dehydrogenase. Merritt and Tomkins[21] reported that reversible oxidation of cyclic secondary alcohols was also catalyzed by liver alcohol dehydrogenase. The interconversion between 3-OH-MHB and 3-keto-MHB was catalyzed by the enzyme in the soluble fraction of the liver,[10] but both DPN and TPN exert the effect upon this reaction.[22] Accordingly, it is interesting to examine what kind of same enzyme is concerning in this reaction.

14) B.B. Brodie, J. Axelrod, J.R. Cooper, L. Gaudette, B.N. La Du, C. Mitoma, S. Udenfriend: Science, **121**, 603 (1955).
15) B.B. Brodie, J.R. Gillette, B.N. La Du : Ann. Rev. Biochem., **27**, 427 (1958).
16) O. Hayaishi : Proc. Intern. Symposium. Enzyme Chem., p. 207 **Tokyo, 1957.**
17) H.S. Mason : Advances in Enzymology, **19**, 79 (1957).
18) Y. Imai, R. Sato : Biochim. et Biophys. Acta, **36**, 571 (1959).
19) *Idem* : *Ibid.*, **42**, 164 (1960).
20) J.R. Gillette : J. Biol. Chem., **234**, 139 (1959).
21) A.D. Merritt, G.M. Tomkins : *Ibid.*, **234**, 2778 (1959).
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The authors are indebted to Dainippon Pharmaceutical Co., Ltd. for a supply of MHB, and also to Smith, Kline and French Laboratories for a donation of SKF 525-A. This work was supported partly by a Grant in aid for Scientific Research provided by the Ministry of Education, to which the authors' thanks are also due.

Summary

An enzyme system in rabbit liver homogenate oxidizes MHB to yield 3-OH-MHB and 3-keto-MHB.

MHB is at first oxidized to 3-OH-MHB by a TPNH-dependent microsomal enzyme system. 3-Keto-MHB are then produced from 3-OH-MHB and both metabolites are interconvertible in the soluble fraction of the liver.

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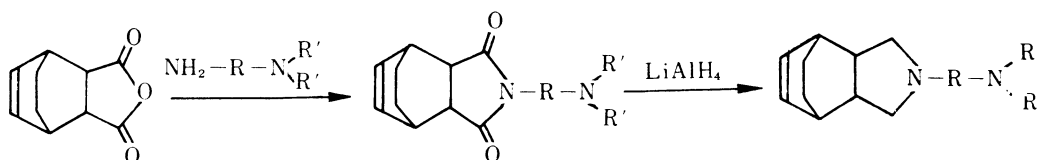
UDC 547.759.07

115. **Manabu Fujimoto et Keiko Okabe** : Nouvelle Méthode de Synthèse des Dérivés d'Ethano-4,7 Polyhydroisoindoline.[*1]

(*Laboratoire de Recherches, Shionogi et Cie., S. A.*[*2])

Nous avons obtenu jusqèu'ici les sels quaternaires d'éthano-4,7 polyhydroisoindoline par le procédé suivant (Schéme 1).



Schème 1.

Le KK-25-S[*3] ou IK-64-S[*4] est connu comme un remède à l'action ganglioplégique, surtout comme un hypotenseur puissant en genre d'hexaméthonium.[2)] Cependant dans ce procédé, on doit toujours employer une série de diamines aliphatiques et l'alanate de lithium comme réducteur, et pour cela, il est peu convenable en se placant du point de vue économique.

Le but de ce travail est d'étudier les méthodes de la préparation des dérivés d'éthano-4,7 polyhydroisoindoline, incluant les KK-25-S et IK-64-S, à partir de l'èthano-4,7 tétrahydro-3a,4,7,7a isoindolinedione-1,3 (I) et d' l'éthano-4,7 perhydroisoindolinedione-1,3 (II).

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*1 La nomenclature de ce série comme des dérivés d'éthano-4,7 isoindole se conforme à la description dans "The Ring Index, RRI. 2936 (1960)." Donc notre noyau hétérocyclique se nommer comme l'éthano-4,7 polyhydroisoindoline.

*2 192, Imafuku, Amagasaki, Hyôgo-ken (藤本 学, 岡部桂子).

*3 Diméthiodure de (diméthylamino-3 propyl)-2 éthano-4,7 tétrahydro-3a,4,7,7a isoindoline.

*4 Diméthiodure d'éthano-4,7 tétrahydro-3a,4,7,7a (pyrrolidino-2 éthyl)-2 isoindoline.

1) K. Takeda, *et al.* : Ann. Rept. Shionogi Research Lab., **10**, 1 (1960).
2) T. Minesita, *et al.* : *Ibid.*, **10**, 15 (1960).