A NEW ANTIBIOTIC, FUMARAMIDMYCIN

II. ISOLATION, STRUCTURE AND SYNTHESES

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A new antibiotic fumaramidmycin produced by *Streptomyces kurssanovii* NR-7GG1 was isolated as colorless crystals. The structure was shown to be N-(phenylacetyl) fumaramide. Starting from fumaramic acid, fumaramidmycin has been synthesized in good yield, in which the key stage involves N-acylated imino ether formation followed by mild acid hydrolysis. Five analogues of fumaramidmycin have also been prepared.

As described in the preceding paper[1], fumaramidmycin is a new metabolite produced by agar cultures of *Streptmyces kurssanovii* NR7GG1, possessing an antimicrobial activity against Gram-positive and Gram-negative bacteria. This paper deals with the isolation, characterization, structure determination and synthesis of fumaramidmycin as well as the preparation of its analogues.

Isolation and Characterization

A schematic representation of the isolation process is shown in Fig. 1. The details are described in Experimental section.

Fumaramidmycin is a colorless crystalline compound melting at 202~203°C with decomposition, $[\alpha]_D^{25}$ 0° (*c* 2, DMSO). It is soluble in DMF and DMSO, but almost insoluble in other organic solvents and water. Fumaramidmycin is very unstable in alkaline solution. The UV, IR, Mass and NMR spectra are shown in Figs. 2, 3, 4 and 5, respectively.

Structure

By elemental analysis the empirical formula of fumaramidmycin was shown to be $C_{12}H_{12}N_2O_3$ (M.W. 232). The molecular weight was confirmed by the M^+ peak in the mass spectrum (Fig. 4). The presence of a benzyl group linked to a nitrogen atom or unsaturated carbon atom, in either case without bearing hydrogen atoms, was indicated by the benzene-ring proton signals at δ 7.15~7.35 (5H) and the isolated methylene proton signal at δ 3.88

* The antibiotic was formerly named and presented as Ro 09-0049 (See Abst. of 95th Ann. Meeting Jap. Soc. Pharm. Sci., 1975).

Fig. 1. Isolation and purification of fumaramidmycin

Agar culture (3.5 liters)
- 1) extracted with methanol
- 2) concentrated

Aqueous solution
- 1) extracted with butyl acetate
- 2) evaporated

Yellowish syrup

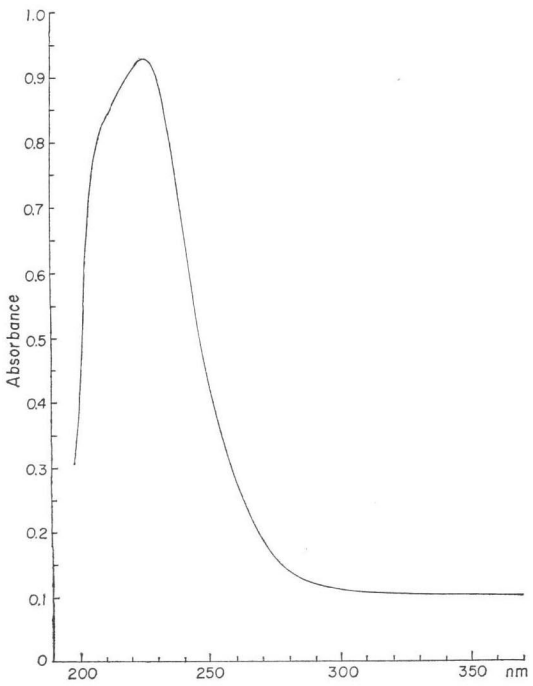
Silica-gel chromatography
- eluted with chloroform - methanol (20:1)

Active fraction
- evaporated

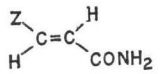
Pale yellow crystals
- recrystallized from chloroform - methanol

Colorless crystals (17 mg)

Fig. 2. UV spectrum of fumaramidmycin in ethanol.



(2H) in the NMR spectrum (Fig. 5), and by the mass peak at *m/e* 91 assignable to tropylium ion. A partial structure shown below was also indicated from the following spectral data:



Z: Heteroatom or unsaturated carbon atom, not bearing hydrogen atom.

i) the UV spectrum in ethanol solution (Fig. 2) shows a maximum at 225 nm (ε 23,400), suggesting the presence of $\alpha\beta$-unsaturated carbonyl group; ii) the IR spectrum (Fig. 3) is consistent with the presence of a primary amide group, as indicated by the bands at 3400, 3200 cm^{-1} (νNH) and 1695 cm^{-1} (amide I); iii) the NMR spectrum shows the presence of two *trans* hydrogens (AB system doublets at δ 6.96 and 7.17, J_{AB}=14.5 Hz) on a conjugated C=C group, two adjacent atoms of which do not bear hydrogens, as well as the presence of a primary amide group (two broad signals at δ 7.86 and 7.42; slowly exchangeable with D_2O (Fig. 5)); iv) a nuclear OVERHAUSER effect was observed between one of the olefinic hydrogens (δ 6.96)

Fig. 3. IR spectrum of fumaramidmycin (KBr).

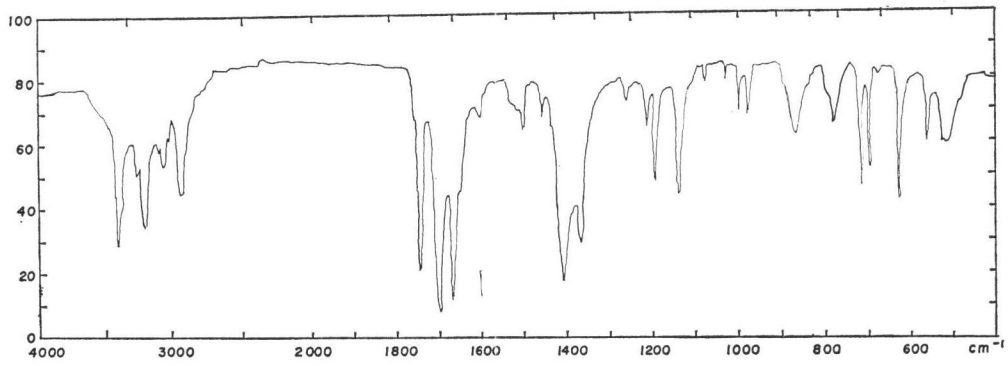


Fig. 4. Mass spectrum of fumaramidmycin (70 eV).

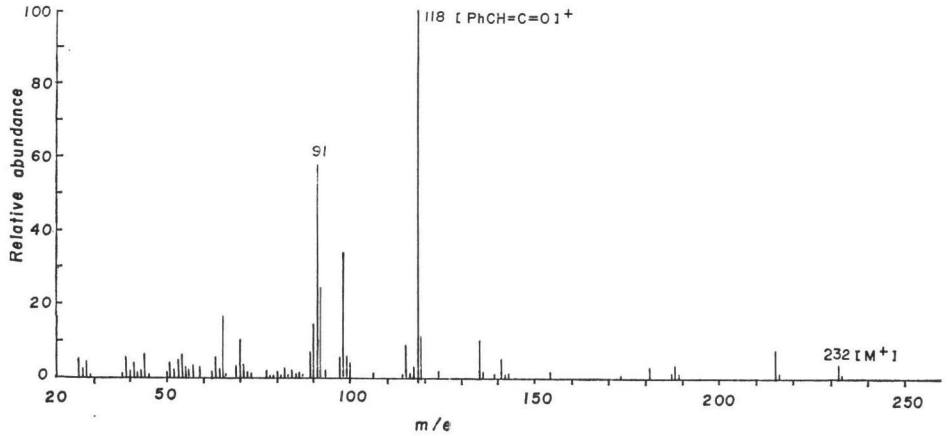
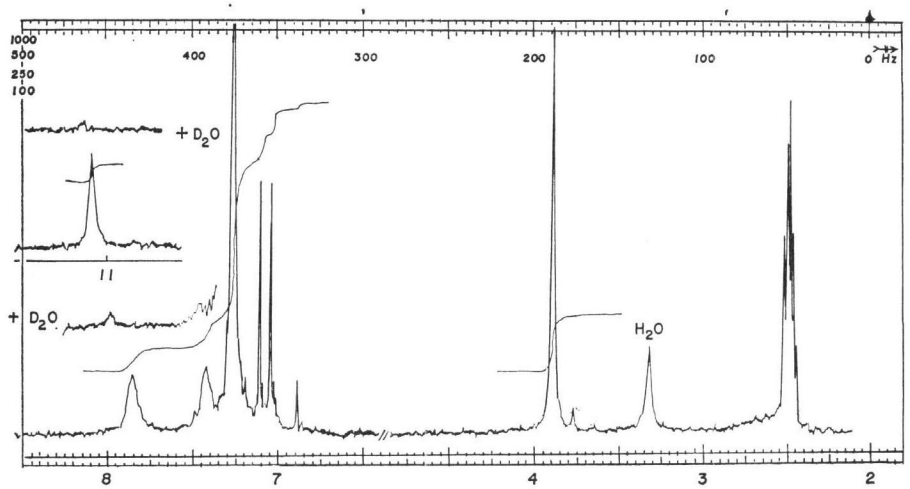
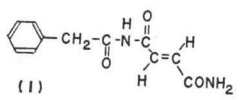


Fig. 5. NMR spectrum of fumaramidmycin in DMSO-d_6 at 100 MH_z.

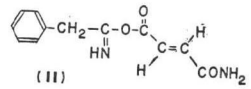


and one of the amide hydrogens (δ 7.86).

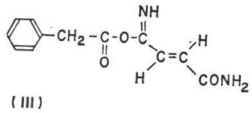
Since the remaining hydrogen (one proton signal at δ 11.09) is exchangeable with D_2O, the followings (I~V) are proposed as the possible structures for fumaramidmycin.



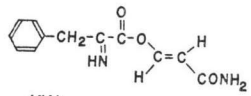
(I)



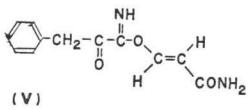
(II)



(III)



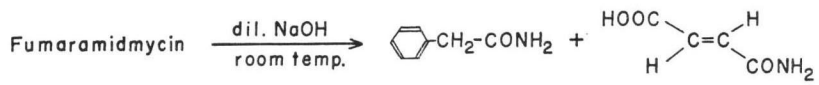
(IV)



(V)

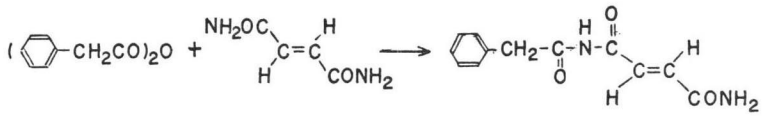
As described above, fumaramidmycin is much more unstable in alkaline solution than in acidic solution. On mild alkaline hydrolysis, fumaramidmycin afforded phenylacetamide and fumramic acid[2] as shown below.

This evidence, together with the well known fact that acyclic isoimides such as **II** or **III** are too unstable* to be isolated[3-6], supports the imide (**I**) as the most probable



structure for fumaramidmycin.

In order to confirm the proposed structure, N-(phenylacetyl)-fumaramide (**I**) was synthesized by treating fumaramide with phenylacetic anhydride following the method of HURD *et al.*[7]



(I)

Although the yield was very low, the product was identical with natural fumaramidmycin with respect to spectral data (UV, IR, NMR, and mass spectra) and the antibacterial activity. Since no compound has been found among reported antibiotics corresponding to these physicochemical characteristics and structure, it was confirmed that fumaramidmycin is a new antibiotic. After we presented the present work orally at the 95th Annual Meeting of Jap. Soc. Pharm. Sci. (Apr. 6, 1975), we were informed that HASEGAWA *et al*** also found the antibiotic independently.

Syntheses of Fumaramidmycin and its Analogues

For reasons described in the preceding paper[1], the microbial process is not practical to obtain sufficient amounts of this antibiotic for the investigation of its biological activities.

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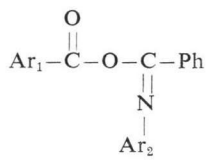
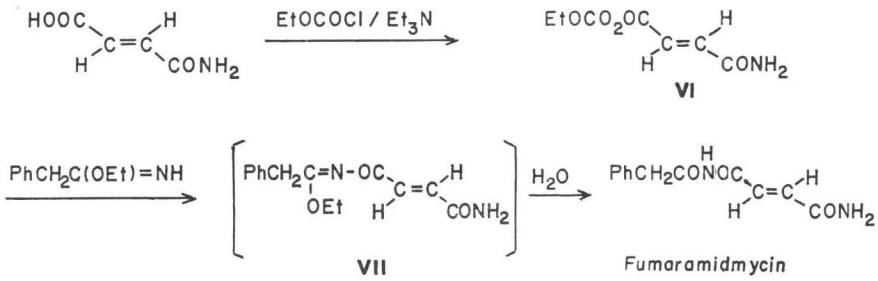
* Although acyclic isoimides with the structure shown below have been found to be sufficiently stable for isolation if Ar_2 contains two nitro groups (*i.e.* 2, 4-dinitrophenyl) to depress the nucleophilicity of the imido nitrogen atom, these compounds rearrange to the corresponding imides (with 1, 3 acyl migration) in benzene or acetonitrile solution even at 42~65°C[6]. Under similar conditions, fumaramidmycin was found to be stable.



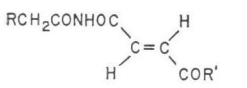
** Takeda Chemical Industries, Ltd.

Therefore, chemical synthesis of the antibiotic and its analogues were attempted.

In spite of its simple structure, several trials to prepare the substance by conventional methods[7,8] for imide synthesis resulted in poor yields. The failure seemed to be due mainly to the instability of the antibiotic. Therefore, we investigated the following synthetic route that appeared to be mild enough for preparing such rather unstable imide.



With the mixed anhydride (**VI**), prepared from fumaramic acid and ethyl chloroformate, ethyl phenylacetimidate was acylated in good yield. The resulting N-acylated imino ether (**VII**) was easily hydrolyzed by dilute hydrochloric acid at 0°C to afford fumaramidmycin.



	R	R'
VIII	Phenyl	NMe_2
IX	p-Methoxyphenyl	NH_2
X	p-Nitrophenyl	NH_2
XI	Phenoxy	NH_2
XII	Phenyl	OEt

The overall yield from fumaramic acid was 60.4%, where the intermediates, **VI** and **VII** were not isolated. In order to examine structure-activity relationship, the following analogues of fumaramidmycin were also prepared in a similar manner. The comparison of their biological properties with that of fumaramidmycin will be described elsewhere.

Experimental

All melting points were obtained by the use of a Yamato MT-1 capillary melting point apparatus and are uncorrected. IR spectra were determined by a Hitachi EPI-G3 grating spectrometer as KBr pellets. UV spectra were measured with a Hitachi 124 spectrometer. NMR spectra were measured with a Varian HA-100 instrument with TMS as the internal standard. Mass spectra were taken with a Hitachi RMU-6E spectrometer. The optical rotation was measured by a Carl Zeiss LEP-A2 photoelectric polarimeter. Thin-layer chromatography (TLC) was performed on silica gel F254 (Merck), spots being visualized with UV light or iodine vapor. Solvents used for TLC were: A, chloroform-methanol (5:1); B, ethanol-water-28% ammonia (20:3:1).

Isolation of Fumaramidmycin

The strain, *Streptomyces kurssanovii* NR7GG1, was inoculated on GGN-agar[1] plates with total area of 3,000 cm^2 (approx. 1-cm thick, total volume 3.5 liters) by streaking at 1-cm intervals, and incubated for 5 days at 27°C. The whole cultured agar including mycelia was cut into small pieces and extracted by stirring three times with 3 liters of methanol. The methanol extracts were concentrated under reduced pressure to a volume of *ca.* 300 ml. The resulting viscous fluid, consisting mainly of water from the agar, was extracted 5 times with 300 ml of butyl acetate. The combined extracts were concentrated to yield yellowish syrup, which was then applied on a column of silica gel (40 g of Wako gel C-200, Wako Co.). After

washing the column with chloroform - methanol (50:1, v/v), the antibacterial activity was eluted with chloroform - methanol (20:1, v/v). The active eluate was evaporated under reduced pressure to give a pale yellow crystalline residue. Recrystallization of the residue from chloroform - methanol yielded 17 mg of fumaramidmycin as colorless crystals.

Anal. Calcd. for $C_{12}H_{12}N_2O_3$: C, 62.06; H, 5.21; N, 12.06.
Found: C, 61.04; H, 5.34; N, 11.94.

The product showed a single spot of Rf 0.65 on TLC (solvent A).

Hydrolysis of Fumaramidmycin

To a solution of fumaramidmycin (9.47 mg in 0.5 ml of DMSO), was added 2 ml of 0.1 N NaOH, and the mixture was stirred for 5 minutes at room temperature. Water (20 ml) was added, and the mixture was extracted with two 20-ml portions of *n*-butyl acetate. The combined butyl acetate extracts, were washed with 10 ml of water and evaporated under reduced pressuse, giving 2.46 mg of a colorless solid. Examination of this product by TLC (solvent A) revealed the presence of at least two materials. The major component (Rf 0.84) was separated by preparative TLC, and was shown to be identical with phenylacetamide by TLC, UV and IR.

The aqueous phase described above was passed through a column of Dowex 50 W×4 (50 ~100 mesh, H-form, 10 ml) and the column was washed with 50 ml of water. The effluent and washing were combined, evaporated under reduced pressure, affording a syrupy residue. TLC examination (solvent B) showed that the major component of this material has the same Rf value (0.65) as that of fumaramic acid. Separation of the major component by preparative TLC yielded 5.21 mg of a colorless solid which was then dissolved in 2 ml of water. The solution was passed through a column of Dowex 50W×4 (5 ml) to remove ammonium ion. The combined effluent and washing (15 ml) was evaporated to give 2.94 mg of a colorless crystalline material, whose UV and IR spectra were identical with those of fumaramic acid.[2)]

Confirmation of the Proposed Structure (**I**) by Synthesis

A finely powdered mixture of 0.01 mole of phenylacetic anhydride (2.54 g) and 0.025 mole of fumaramide (2.86 g) was heated at 130~135° for 5 minutes. Two drops of concentrated sulfuric acid were added to the resulting slurry, which was then heated again at 140~150°C for 10 minutes with stirring. After standing for 15 minutes at room temperature, the reaction mixture solidified. The solid was triturated well with 50 ml of cold water. The resulting suspension was filtered and washed with 40 ml of DMSO. The filtrate combined with washings was diluted with 100 ml of cold water and extracted with four 150-ml portions of butyl acetate. The combined butyl acetate extracts were washed with two 100-ml portions of water and then evaporated under reduced pressure. To the brown residue was added 5 ml of chloroform and the mixture was applied on a silica gel column (Wako gel C-200, 2×40 cm), which was then developed successively with chloroform, chloroform - methanol (50:1), and chloroform - methanol (20:1). The fractions eluted with chloroform - methanol (20:1) were active against *Escherichia coli* K-12. Such fractions were combined and concentrated under reduced pressure to a volume of about 1 ml. After standing in a refrigerator overnight, the precipitated solid was collected by filtration and washed with a small amount of cold ethanol and then dried. TLC examination (solvent A) of the product revealed the presence of at least three components and the Rf value (0.65) of the major component was identical with that of natural fumaramidmycin. Further purification of the major component by preparative TLC (solvent A) gave 9.4 mg (0.4 % yield) of a colorless crystalline solid which was identical with natural fumaramidmycin with respect to UV, IR, NMR, mobilities on TLC, and biological activity.

Synthesis of N-(Phenylacetyl) fumaramide, Fumaramidmycin (**I**)

A solution containing 17.3 g (0.15 mol) of fumaramic acid and 21 ml (0.15 mol) of triethylamine in 150 ml of DMF was cooled to −20°C. A solution of 16.2 g (0.15 mol) of ethyl chloroformate in 75 ml of ether was added dropwise with stirring and resulting suspension was stirred for 45 minutes at −20°C. Into the suspension was added quickly a well cooled solution

(−20°C) of ethyl phenylacetimidate base* (0.15 mol) in DMF. The mixture was stirred for 3 hours at −10° to 0°C. The separated triethylamine hydrochloride was removed by filtration and the filtrate was evaporated at 0.1 Torr/25°. The residual oil was suspended in 200 ml of water, cooled to 0°C and brought to pH 1.5 with 1N hydrochloric acid under stirring. Immediately, crystals appeared and the suspension was stirred further for 30 minutes at 0°C. The crystals were collected, washed with water to neutrality, then washed successively with ethyl acetate and ether. Recrystallization from DMF-ethanol and further recrystallization from DMF-water afforded 21 g (60.2 % yield) of fumaramidmycin as colorless crystals.

N, N-Dimethyl-N'-(phenylacetyl) fumaramide (**VIII**)

This compound was prepared in an analogous manner from N, N-dimethylfumaramic acid and ethyl phenylacetimidate: mp from 182°C (dec). NMR (DMSO-d_6) δ 2.91 and 3.09 (each 3H, s, NMe_2), 3.90 (2H, s, CH_2), 7.10 and 7.50 (each 1H, AB system d, J=15 Hz, *trans* olefinic H's), 7.32 (5H, s, arom. H's) and 11.20 (1H, s, imide H).

N-[(*p*-Methoxyphenyl) acetyl] fumaramide (**IX**)

This compound was prepared in an analogous manner from fumaramic acid and ethyl *p*-methoxyphenylacetimidate: mp from 206°C (dec); NMR (DMSO-d_6) δ 3.77 (3H, s, OMe), 3.85 (2H, s, CH_2), 7.51 and 7.91 (each 1H, broad s, $CONH_2$) and 11.20 (1H, s, imide H).

N-[(*p*-Nitrophenyl) acetyl] fumaramide (**X**)

This compound was prepared in an analogous manner from fumaramic acid and ethyl *p*-nitrophenylacetimidate: mp from 204°C (dec); NMR (DMSO-d_6) δ 4.15 (2H, s, CH_2), 7.10 (2H, s, olefinic H's), 7.55 and 8.17 (each 2H, A_2B_2 system d, J=9 Hz, arom. H's), 7.91 (1H, broad s, one of amide H's) and 11.27 (1H, s, imide H).

N-(Phenoxyacetyl) fumaramide (**XI**)

This compound was prepared in an analgous manner from fumaramic acid and ethyl phenoxyacetimidate: mp 188~190° (dec); NMR δ 5.00 (2H, s, CH_2), 7.41 and 7.90 (each 1H, broad s, $CONH_2$) and 11.28 (1H, s, imide H).

Ethyl N-(Phenylacetyl) fumaramate (**XII**)

This compound was prepared in a similar manner from ethyl 3-(chloroformyl) acrylate and ethyl phenylacetimidate: mp 102°C; NMR (DMSO-d_6) δ 1.24 (3H, t, J=7Hz, CH_2CH_3), 3.89 (2H, s, Ar-CH_2), 4.21 (2H, q, J=7Hz, CH_2CH_3), 6.71 and 7.40 (each 1H, AB system d, J=16Hz, *trans* olefinic H's), 7.30 (5H, s, arom. H's) and 11.27 (1H, s, imide H).

Acknowledgement

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References

1) MARUYAMA, H.B.; Y. SUHARA, J. WATANABE, Y. MAESHIMA, N. SHIMIZU, M. HAMADA, H. FUJIMOTO & K. TAKANO: A new antibiotic, fumaramidmycin I. Production, biological properties and characterization of producer strain. J. Antibiotics 28: 636~647, 1975
2) TALLEY, E.A.; T.J. FITZPATRICK & W.L. PORTER: Formation of fumaramic acid from asparagine in phosphate buffer. J. Amer. Chem. Soc. 81: 174~175, 1959
3) MUMM, O.; H. HESSE & H. VOLQUARTZ: Diacylamides. Ber. 48: 379~391, 1915
4) STEVENS, C.L. & M.E. MUNK: Nitrogen analogues of ketenes. IV. Reaction with carboxylic

* The imidate base was prepared from its hydrochloride (29.9 g, 0.15 mol) by treating with an equivalent amount of triethylamine (21 ml) in 150 ml of DMF at 5~0°C.

acids. J. Amer. Chem. Soc. 80: 4065~4069, 1958
5) CRAMER, S. & K. BAER: Imino ethers, IV. Reaction of imino chlorides with carboxylic acids. Ber. 93: 1231~1236, 1960
6) CURTIN, D. Y. & L. L. MILLER: 1, 3 Acyl migrations in unsaturated triad (allyloid) systems. Rearrangements of N-(2, 4-dinitrophenyl) benzimidoyl benzoates. J. Amer. Chem. Soc. 89: 637~645, 1967
7) HURD, C.D. & A.G. PRAPAS: Preparation of acyclic imides. J. Org. Chem. 24: 388~392, 1959
8) WHEELER, O.H. & O. ROSADO: Chemistry of imidic compounds. pp. 337~352. In: The chemistry of amides. Ed. J. Zabicky, Interscience Publishers, 1970