SYNTHESIS AND ANTIBACTERIAL ACTIVITY OF ASPARAGINE DERIVATIVES OF AMINOBENZYLPENICILLIN[1]

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In order to improve the antibacterial activity of aminobenzylpenicillin, penicillin derivatives having an asparagine moiety in the 6-acyl side chain (**11a**~**g**, **12a**, **b**, **f**, **g**) were synthesized. The structure-activity relationship of new penicillins, N^4-alkyl-asparaginylaminobenzylpenicillins, was investigated.

N^4-Methyl-D-asparaginylamoxicillin (**11a**), TA-058, was found to possess a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria. In acute toxicity, TA-058 showed good tolerance in mice ($LD_{50} > 10$ g/kg, i.v.).

Recently, a number of new type of ampicillin (ABPC) and amoxicillin (AMPC) derivatives, piperacillin[2], azlocillin[3], apalcillin[4] and PL-385[5] have been reported to possess stronger antibacterial activity than that of ABPC and AMPC against Gram-negative bacteria. A common structural feature of these penicillins is the presence of a heterocyclic ring in their side chains. We planned to make a new series of penicillin analogs, in which the asparagine (Asn) moiety was incorporated in place of heterocycles, and various asparagine derivatives of aminobenzylpenicillin were synthesized as shown in Table 1. Among the compounds prepared, N^4-methyl-D-asparaginylamoxicillin (**11a**), TA-058*, showed the most potent activity against Gram-positive and Gram-negative bacteria.

This paper describes the preparation and antibacterial activity of TA-058 and the related compounds.

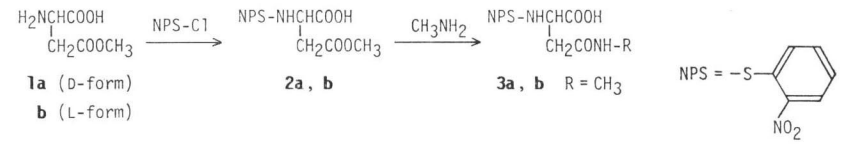
Chemistry

The most important problem during this work was the selection of an appropriate protecting group for the NH_2 group in amino acids. *o*-Nitrophenylsulfenyl (NPS) group was found to be suitable for all the compounds.

Synthesis of N^4-Alkyl-NPS-Asn (**3a**~**e**)

3a (**3b**) were obtained by the reaction of **2a** (**2b**) with methylamine (Chart 1). In other cases (R=

Chart 1.



* N^4-Methyl-D-asparaginyl amoxicillin (**11a**) is currently under clinical trials in Japan under the code name of TA-058.

Chart 2.

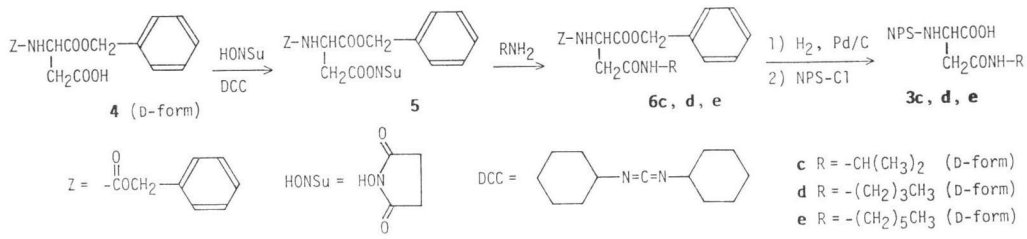
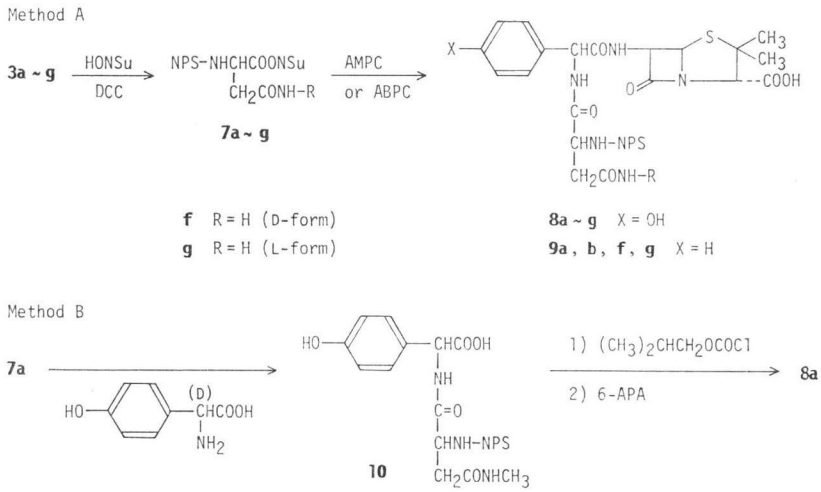


Chart 3.



*iso*Pr, *n*-Bu, *n*-He), **3c ~ e** were prepared by another method as shown in Chart 2.

Condensation of N^4-Alkyl-NPS-Asn and AMPC or ABPC

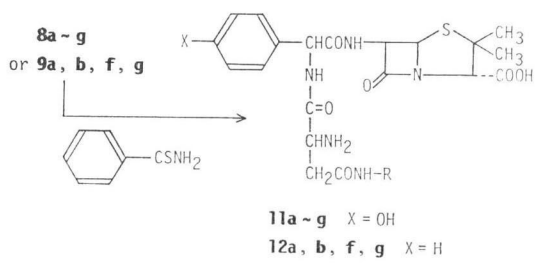
The coupling reaction of N^4-alkyl-NPS-Asn (**3a ~ g**) with AMPC or ABPC was readily accomplished by the active ester method (method A in Chart 3) and **8a ~ g** and **9a**, **b**, **f**, **g** were obtained as yellow crystals or caramels. Alternatively, **8a** could be obtained by the fragment condensation of NPS-dipeptide (**10**) with 6-aminopenicillanic acid (6-APA) by the mixed anhydride method (method B in Chart 3).

No racemization of the *p*-hydroxyphenylglycine moiety was observed in the case of method A, but lightly the racemization occurred by the method B.

Removal of NPS Group with Thiobenzamide

Many procedures for the removal of the NPS protecting group have been reported. ZERVAS and his co-workers[6] reported the use of HCl in an organic solvent. KESSLER and ISELIN,[7] and TUN-KYI[8] investigated the use of thiols in the presence of acetic acid. MEIENHOFER[9] suggested the use of RANEY nickel. PODUŠKA *et al.*[10] showed that sulfonic acid imides were suitable reagents.

Chart 4.



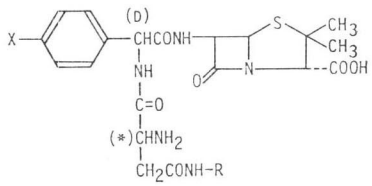
After an extensive screening of the reagents which might effect the removal of the NPS group, we found that thiobenzamide was an efficient reagent which did not affect the β-lactam ring. Selective removal of the NPS group of the compounds (**8a**~**g**, **9a**, **b** **f**, **g**) was effected smoothly by treatment with thiobenzamide under neutral conditions as described in Experimental. The final products (**11a**~**g**, **12a**, **b**, **f**, **g**) were obtained as colorless or pale yellow powders after lyophilization (Chart 4).

Antibacterial Activity

TA-058 (**11a**), a representative of the series studied, shows broad antibacterial activity against Gram-positive and Gram-negative bacteria. Antibacterial activity of the eleven penicillins synthesized is shown in Table 1.

All of the D-isomers in the asparagine moiety (**11a**, **11f**, **12a**, **12f**) were more active than the corresponding L-isomers (**11b**, **11g**, **12b**, **12g**) against *Escherichia coli* and *Pseudomonas aeruginosa*. However, the activity of the D-isomers against *Staphylococcus aureus* was almost equal to that of the L-isomers. As for the substituent (X) on the benzene ring, AMPC series (X=OH) were slightly more active than the corresponding ABPC series (X=H) against *E. coli*. *N*-Alkyl amides (**11a**~**e**, **12a**, **b**) of the asparagine derivatives exhibited almost equal activity against *S. aureus*. In contrast with this behavior to *S. aureus*, the chain length of alkyl groups greatly affected the activity against Gram-negative bacteria. In general, activity of asparagine derivatives against Gram-positive bacteria was weaker than that of ABPC and AMPC, but equal to that of sulbenicillin (SBPC).

Table 1. Antibacterial activity of Asn derivatives of ABPC and AMPC.



No.	X	R	*	MIC (μg/ml)		
				S. aureus FDA 209P JC-1	*E. coli* NIHJ JC-2	*P. aeruginosa* No. 12
11a	OH	CH_3	D	0.78	0.19	6.25
11b	OH	CH_3	L	0.78	25	12.5
11c	OH	$CH(CH_3)_2$	D	0.78	6.25	25
11d	OH	$(CH_2)_3CH_3$	D	0.78	3.13	25
11e	OH	$(CH_2)_5CH_3$	D	0.39	25	25
11f	OH	H	D	0.78	3.13	25
11g	OH	H	L	1.56	25	>100
12a	H	CH_3	D	0.78	6.25	12.5
12b	H	CH_3	L	0.39	25	50
12f	H	H	D	3.13	6.25	6.25
12g	H	H	L	3.13	25	25
ABPC				0.03	3.13	>100
AMPC				0.05	6.25	>100
SBPC				1.56	6.25	25

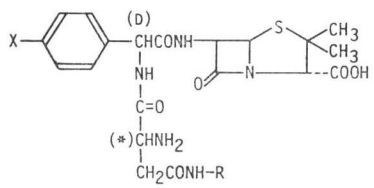
MIC was assayed by the agar dilution method and inoculum size was one loopful of 10^6 CFU/ml.

Table 2. Protective effects of TA-058 and other penicillins (single i.m. injection) on various experimental infections in mice.

Test strain challeng dose (cells/mouse)	ED_{50} (mg/kg) (MIC, μg/ml)		
	TA-058	CBPC	ABPC
Staphylococcus aureus Smith 1.0×10^3 (100 LD_{50})	4.6 (1.56)	18.7 (0.39)	0.1 (<0.19)
Escherichia coli 1346 9.5×10^2 (100 LD_{50})	1.0 (<0.19)	28.5 (1.56)	4.6 (0.39)
Proteus vulgaris TPRL-10820 4.6×10^5 (100 LD_{50})	21.6 (0.39)	126 (1.56)	27.6 (0.39)
Pseudomonas aeruginosa No. 12 1.3×10^4 (10 LD_{50})	35.8 (12.5)	410 (50)	NT (>400)

Drugs were administered intramuscularly 1 hour after infection.
NT: not tested

Table 3. Acute toxicity of TA-058 and related compounds.



No.	X	R	*	LD_{50} (mg/kg, mice, i.v.)
11a	OH	CH_3	D	>10,000
11b	OH	CH_3	L	5,475
11c	OH	$CH(CH_3)_2$	D	6,000
11f	OH	H	D	4,610
11g	OH	H	L	3,840
12a	H	CH_3	D	2,280
12b	H	CH_3	L	NT
12f	H	H	D	2,310
12g	H	H	L	2,310

NT: not tested.

The protective effects of TA-058 were tested in mice infected with *S. aureus*, *E. coli*, *P. vulgaris* or *P. aeruginosa*. Table 2 shows the ED_{50} (mg/kg) of TA-058, carbenicillin (CBPC) and ABPC on these experimental infections.

It is noteworthy that the superior protective effect was found with TA-058 as compared with CBPC, although this compound was weaker than CBPC *in vitro* activity against *S. aureus*. Furthermore, TA-058 was considerably more active than CBPC against Gram-negative bacteria both *in vitro* and *in vivo*. Compared with ABPC, TA-058 was more active in the protective effects against Gram-negative bacteria, but less active against *S. aureus*.

Acute toxicity of the asparagine derivatives of aminobenzylpenicillin in mice is shown in Table 3. All the compounds of the AMPC series (X=OH) were found to exhibit a low toxicity comparing with the ABPC series. In particular the acute toxicity of TA-058 is very low (LD_{50} > 10 g/kg, i.v.).

Further detailed data regarding antibacterial, pharmacokinetic and toxicological aspects of TA-058, which is currently under clinical trials, will be reported elsewhere.

Experimental

All melting points are uncorrected. Infrared spectra were recorded with a Hitachi 215 spectrometer. NMR spectra were measured with JEOL FX-100 NMR spectrometer using DSS as an internal standard. The optical rotations were measured with a Jasco DIP-180 polarimeter.

NPS-D-Asp(OMe) (**2a**)

D-Asp(OMe)·HCl (**1a**, 3.16 g) in H_2O (24 ml) was neutralized with K_2CO_3, and then THF (20 ml) was added. *o*-Nitrophenylsulfenylchloride (3.70 g) was added to the solution, with vigorous stirring at 5 to 10°C. During the reaction, the mixture was kept slightly alkaline (pH 8) with K_2CO_3. Water was added to the reaction mixture, and insoluble materials were filtered off. The filtrate was washed with AcOEt, acidified with citric acid and then extracted with AcOEt. The AcOEt extracts were washed with water, and dried on $MgSO_4$. After evaporation of the solvent, the resulting solids were collected by suction and washed with benzene to give **2a** (3.16 g) as yellow needles: mp 86~87°C, $[\alpha]_D^{20}$ +65.7° (*c* 1, THF).

NPS-L-Asp (OMe) (**2b**)

2b was similarly prepared from L-Asp(OMe)·HCl (**1b**) by the method as described for **2a**: mp 87~88°C, $[\alpha]_D^{20}$ −66.2° (*c* 1, THF).

N^4-Me-NPS-D-Asn (**3a**)

2a (3.80 g) was dissolved in MeOH (10 ml). An aqueous 40% methylamine solution (10 ml) was added to the solution at 0 to 5°C. The mixture was stirred at room temperature for 16 hours. The reaction mixture was evaporated below 40°C under reduced pressure to remove the solvent. The residue was dissolved in water and washed with AcOEt. The aqueous layer was adjusted to pH 3 with an aqueous 10% citric acid solution, and extracted with AcOEt. The extract was washed with water, dried on $MgSO_4$ and then evaporated below 40°C to remove the solvent. Benzene was added to the residue obtained, and the precipitated crystals were collected by the filtration to give **3a** (2.70 g) as yellow needles: mp 134~136°C, $[\alpha]_D^{20}$ +64.7° (*c* 1, THF).

N^4-Me-NPS-L-Asn (**3b**)

3b was similarly prepared from NPS-L-Asp(OMe) (**2b**) by the method as described for **3a**, yellow needles: mp 135~136°C, $[\alpha]_D^{20}$ −65.0° (*c* 1, THF).

N^4-*iso*Pr-Z-D-Asn-OBzl (**6c**)

To a solution of Z-D-Asp-OBzl (**4**, 12.0 g) in THF (200 ml) were added DCC (7.62 g) and *N*-hydroxysuccinimide (4.25 g) under ice cooling. After stirring at 0 to 5°C for 4 hours, precipitates were filtered off. Then, the filtrate was evaporated to remove the solvent, and crude Z-D-Asp(ONSu)-OBzl (**5**, 16.3g) was obtained as a pale yellow caramel.

To a solution of the crude active ester (**5**, 5.40 g) in DMF (20 ml) was added isopropylamine (1.3 g) at 5°C. After stirring at the room temperature for 1 hour, the mixture was poured into water and extracted with AcOEt. The extract was washed with water, dried on $MgSO_4$, and evaporated to remove the solvent. The residue thus obtained was washed with *iso*-propyl ether to give **6c** (3.60 g) as colorless crystals: mp 124~127°C, IR (Nujol) 3340, 3320, 1755, 1685, 1635 cm^{-1}.

N^4-*iso*Pr-NPS-D-Asn (**3c**)

To a suspension of **6c** (3.60 g) in MeOH (60 ml) and 10% HCl (5 ml) was added 10% Pd/C (0.60 g). The solution was shaken at room temperature in hydrogen gas for 1 hour under atmospheric pressure. After the reaction, insoluble materials were removed by filtration, the filtrate was evaporated under reduced pressure to remove the solvent, and crude N^4-*iso*Pr-D-Asn·HCl (2.00 g) was obtained as a colorless caramel.

Crude N^4-*iso*Pr-D-Asn·HCl (2.00 g) in H_2O (20 ml) was neutralized with K_2CO_3, and THF (20 ml) was added. *o*-Nitrophenylsulfenylchloride (2.20 g) was added to the solution with vigorous stirring at 5 to 10°C. During the reaction, the mixture was kept slightly alkaline (pH 8) with K_2CO_3. After the reaction, the solution was worked up as described above for the preparation of **2a** to give **3c** (2.3 g) as yellow needles: mp 146~147°C (dec.), IR(Nujol) 3385, 3220, 1710, 1640 cm^{-1}.

3d and **3e** were obtained from **6d** and **6e** by treatment similar to that described for **3c** (Table 4).

NPS-D-Asn (**3f**)

3f was prepared from D-Asn by the method reported by ZERVAS, *et al.*[6]: mp 164~165°C, $[\alpha]_D^{20}$ +117° (*c* 1, DMF).

strain produced strobilurins A and B as the sole antibiotics like the other species of this genus[7].

Marasmic acid exhibits a broad antimicrobial spectrum. As already determined by KAVANAGH *et al.*[2] and confirmed with our test-organisms, marasmic acid inhibits Gram-negative and Gram-positive bacteria at concentrations of $1 \sim 10$ μg/ml. In addition, we found a pronounced inhibition of the phytopathogenic fungi *Ceratocystis fimbriata, Botrytis cinerea* and *Sclerotinia fructigena* by marasmic acid at $10 \sim 50$ μg per disc in the plate diffusion assay. $NaBH_4$-reduction[3] of the molecule led to the derivative **2** which no longer showed antibiotic activity. In contrast, methylation with diazomethane[8] resulted in methyl marasmate (**3**) and no loss in antimicrobial activity. We therefore assume that the α,β-unsaturated aldehyde or in the case of **3** the dialdehyde group is essential for the antibiotic activity of marasmic acid and methyl marasmate. Interestingly the addition of cysteine has no effect on the antimicrobial and cytotoxic activities of marasmic acid.

The effect of marasmic acid and methyl marasmate on eucaryotic macromolecular syntheses was tested in cells of the ascitic form of Ehrlich carcinoma (ECA). Both compounds strongly interfere with the incorporation of thymidine and uridine into DNA and RNA, respectively. At a concentration of 3 μg/ml of compound **1** or **3** DNA and RNA syntheses were almost completely inhibited, whereas protein synthesis was much less impaired.

As shown in Fig. 1 the same concentration of marasmic acid did not inhibit the transport of the precursors thymidine and uridine into the cells in a test system as described earlier[8], thus pointing to a direct interference of marasmic acid with nucleic acid synthesis. The same effect, a preferential inhibition of DNA and RNA syntheses without interference with transport of the precursors was found with *Bacillus brevis* cells. DNA and RNA syntheses were directly tested in toluene-treated *B. brevis* cells and in isolated nuclei from ECA cells. As shown in Table 1 marasmic acid inhibits the incorporation of dTMP into DNA at relatively high concentrations. The cell density in this test, however, was about two orders of magnitude higher as compared to the cell density used for the evaluation of the minimal inhibitory concentration (for *B. brevis* 2 μg/ml in the serial dilution assay).

The effect of marasmic acid on eucaryotic RNA synthesis is more pronounced. As shown in Table

Fig. 1. Comparison of uptake and incorporation of [^{14}C]leucine, [^{14}C]uridine, and [^{14}C]thymidine in ECA cells.

The experiment was carried out according to reference 8. The assays containing 3 μg/ml marasmic acid were compared to controls containing no antibiotic (=100%).

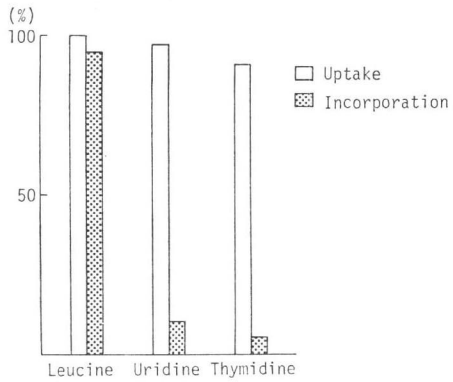


Table 1. Effect of marasmic acid on DNA synthesis in toluene-treated cells of *Bacillus brevis* (1.6×10^8 cells/ml).

Marasmic acid (μg/ml)	Incorporation of [^{3}H]dTMP (pmole)	% of control
0	3.75	100
100	2.06	55

Table 2. Effect of marasmic acid of RNA synthesis in isolated nuclei of ECA cells.

Antibiotic added (μg/ml)	Incorporation of [^{3}H]UMP (pmole)	% of control
None	2.14	100
α-Amanitin 2	0.87	41
Marasmic acid 8	1.22	57
80	0.59	28

N^4-Me-D-Asn-AMPC (**11a**, TA-058)

8a (6.64 g) and thiobenzamide (4.30 g) were dissolved in a mixture of THF (12 ml) and EtOH (36 ml). The solution was stirred at room temperature for 2 hours. The reaction mixture was evaporated below 35°C under reduced pressure to remove the solvent. The residue was triturated in THF, and pale yellow precipitates were collected by filtration. The precipitate thus obtained was dissolved in water. Insoluble materials were filtered off. The filtrate was chromatographed on a Diaion HP-20 (manufactured by Mitsubishi Chem. Ind. Ltd.) column. Elution with water and lyophilization of the eluate gave **11a** (2.00 g) as a colorless powder: mp 198~201°C (dec.), IR (KBr) 3300~3100 (broad), 1758, 1660, 1600, 1540~1510 cm^{-1}, NMR (100 MHz, D_2O) 1.42 (3H, s, CH_3), 1.46 (3H, s, CH_3), 2.76 (3H, s, N-CH_3), 2.89~2.99 (2H, m, $-CH_2-$), 4.15 (1H, s, C_3–H), 4.36 (1H, m, C*H*–NH_2), 5.40 (1H, d, C_5–H), 5.46 (1H, d, C_6–H), 5.40 (1H, s, $-\overset{|}{C}H-\langle\bigcirc\rangle-OH$), 6.93 (2H, d, aromatic proton), 7.34 ppm (2H, d, aromatic proton), $[\alpha]_D^{20}+179.3°$ (*c* 0.5, H_2O).

Anal. Calcd. for $C_{21}H_{27}O_7N_5S\cdot 3H_2O$: C 46.25, H 6.07, N 12.79, S 5.86
Found: C 46.16, H 5.92, N 12.78, S 5.83.

N^4-Me-L-Asn-AMPC (**11b**)

8b (3.30 g) and thiobenzamide (2.22 g) were dissolved in a mixture of MeOH (50 ml) and THF (20 ml). The solution was stirred at room temperature for 40 minutes, and then evaporated below 30°C under reduced pressure to remove the solvent. The residue was triturated in THF (40 ml), and a precipitate was collected by filtration. This was dissolved in 30% aqueous THF (30 ml), and washed three times with a mixture of AcOEt and THF (1: 1). Then the aqueous layer was freeze-dried to give **11b** (1.86 g) as pale yellow powder: mp 197~200°C (dec.), IR (Nujol) 3250 (broad), 1760, 1650, 1600 cm^{-1}.

Other asparaginylamoxicillin derivatives (**11c~g**) were obtained by treatment similar to that described above for the preparation of **11b**. Melting point and IR spectral data of these compounds are given in Table 7.

Asparaginylampicillin Derivatives (**12a, 12b, 12f, 12g**)

Asparaginylampicillin derivatives were prepared from ABPC by the active ester method in the same manner as described for the preparation of asparaginylamoxicillin derivatives (Table 8).

Table 7.

Compound	mp (°C, dec.)	IR (Nujol, cm^{-1})
11c	198~200	3250 (broad), 1760, 1650, 1595
11d	188~191	3270 (broad), 1760, 1650, 1600
11e	186~189	3250 (broad), 1765, 1655, 1595
11f	213~215	3300 (broad), 1760, 1660, 1590
11g	190~197	3325 (broad), 1765, 1660, 1600

Table 8.

Compound	mp (°C, dec.)	IR (Nujol, cm^{-1})
9a	145~147	3250, 1780, 1725, 1630
9b	144~146	3250, 1770, 1730, 1635
9f	115~118	3300, 1770, 1725, 1665
9g	123~125	3320, 1770, 1725, 1660
12a	193~195	3275 (broad), 1760, 1650, 1600
12b	191~193	3270 (broad), 1760, 1650, 1605
12f	216~218	3250 (broad), 1760, 1660, 1600
12g	185~188	3255 (broad), 1760, 1665, 1600

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