Synthesis of Muramyl Dipeptide Analogs with Enhanced Adjuvant Activity[1)]

Shigeru KOBAYASHI,* Tsunehiko FUKUDA, Hidefumi YUKIMASA, Masahiko FUJINO, Ichiro AZUMA,† and Yuichi YAMAMURA††

Chemical Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532

† *Institute of Immunological Science, Hokkaido University, Kita-ku, Sapporo 060*

†† *The Third Department of Internal Medicine, Osaka University, Fukushima-ku, Osaka 553*

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N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and thirteen new analogs were synthesized by the conventional organic chemical procedure using dicyclohexylcarbodiimide-*N*-hydroxy-5-norbornene-2,3-dicarboximide as a coupling agent. Their ability to induce delayed-type hypersensitivity to *N*-acetyl-3-(4-arsonophenylazo)-L-tyrosine in guinea pigs was assayed. The results indicate that the presence of an α-amino acid adjacent to the lactic acid moiety in MDP is important for the high biological activity, the valine analog having the most favorable effect. The structure-activity relationship is discussed.

Freund's complete adjuvant (FCA) is a most widely used adjuvant consisting of heat-killed mycobacterial cells in mineral oil.[2)] Ellouz *et al.*[3)] and Kotani *et al.*[4)] independently elucidated that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) (**9a**) is the minimal structure responsible for the activity elicited by peptidoglycan of the bacterial cell walls. MDP can be substituted for mycobacterial cells in FCA to potentiate both B cell- and T cell-mediated immune responses.[5)] Recently, MDP has been found to have biological activities such as enhancement of the nonspecific resistance in mice infected by *Klebsiella pneumoniae*,[6)] stimulation of the thymidine uptake in mouse spleen cell cultures,[7)] and the activation of reticuloendothelial system[8)] and macrophages *in vitro*.[9)]

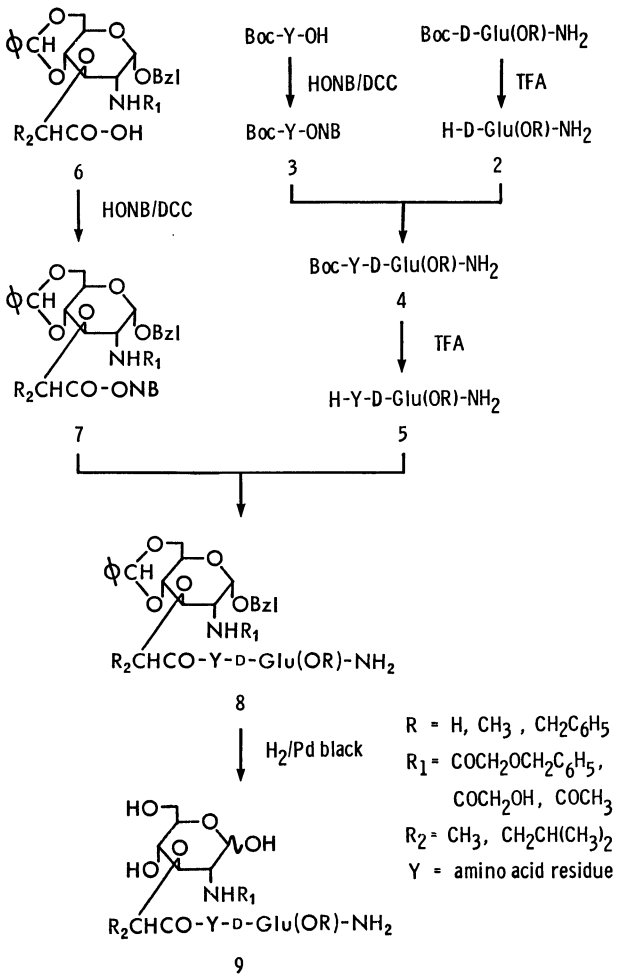
In view of possible clinical applications of immunomodulators, it is important to prepare MDP analogs with enhanced activities. In this report, we describe the synthesis and immunological activities of 13 new MDP analogs (Table 3). MDP has also been synthesized for the sake of comparison in our biological system.

Chemistry

MDP and its analogs were synthesized by the conventional organic chemical procedure using dicyclohexylcarbodiimide (DCC)-*N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB)[10)] as a coupling agent at each step. The *t*-butoxycarbonyl (Boc) group was used for α-amino protection and the benzyl group for side chain hydroxyl and carboxyl protection. The Boc group was removed with trifluoroacetic acid prior to each coupling. The final deprotection of all the protecting groups was effected by catalytic hydrogenation.[11)] Details of the synthesis are summarized in Scheme 1.

The protected dipeptides (**4**) were synthesized by coupling D-isoglutamine benzyl (**2a**) or methyl (**2b**) ester with Boc-amino acid HONB ester (**3**). The dipeptides (**4**) thus obtained are given together with their physicochemical properties in Table 1.

The protected glycopeptides (**8**) were prepared by coupling suitably protected dipeptides (**5**) with protected muramic acid derivatives, such as benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[1-(*R*)-carboxyethyl]-2-deoxy-α-D-glucopyranoside (**6a**), the (*S*)-isomer (**6b**), benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[1-(*R*)-carboxyisopentyl]-2-deoxy-α-D-glucopyranoside (**6c**), or benzyl 4,6-*O*-benzylidene-2-benzyloxyacetamido-3-*O*-[1-(*R*)-carboxyethyl]-2-deoxy-α-D-glucopyranoside (**6d**). These protected muramic acids (**6**) were converted into the HONB active esters (**7**) for coupling.



Scheme 1.

Protected *N*-acetylmuramic acid (**6a**) was prepared by the method of Flowers and Jeanloz[12)] and protected

Table 1. Yields and physicochemical properties of dipeptides, Boc-Y-D-Glu(OR)-NH_2 **4**

Compd	Y	R	Yield/%	Mp/°C	$[\alpha]_D$ (c 0.5) (temp, solvent)	Formula	Found (Calcd)[%] C	H	N
4a	L-Ala	$CH_2C_6H_5$	83.2	140—141[a]	+8.3° (20, MeOH)[a]	$C_{20}H_{29}N_3O_6$	59.41 (58.95)	7.23 7.17	10.25 10.31)
4b	β-Ala	$CH_2C_6H_5$	81.0	148—149	+11.4° (20, MeOH)	$C_{20}H_{29}N_3O_6$	58.96 (58.95)	7.18 7.17	10.24 10.31)
4c	Sar	$CH_2C_6H_5$	89.3	110—112	+4.0° (20, MeOH)	$C_{20}H_{29}N_3O_6$	50.06 (58.95)	7.39 7.17	10.10 10.31)
4d	Aib[b]	$CH_2C_6H_5$	70.2	112—113	+13.2° (27, EtOH)	$C_{21}H_{31}N_3O_6$	59.81 (59.84)	7.76 7.41	9.84 9.97)
4e	Pro	$CH_2C_6H_5$	72.3	155—156	−12.6° (27, DMF)	$C_{22}H_{31}N_3O_6$	61.04 (60.96)	7.43 7.21	9.67 9.69)
4f	L-Thr(OBzl)	$CH_2C_6H_5$	64.5	115—117	+6.0° (27. EtOH)	$C_{28}H_{37}N_3O_7$	63.92 (63.74)	7.14 7.07	7.83 7.96)
4g	L-Val	$CH_2C_6H_5$	81.2	148	+8.9° (25, DMF)	$C_{22}H_{33}N_3O_6$	60.53 (60.67)	7.74 7.64	9.54 9.65)
4h	L-Val	CH_3	84.3	117—119	+8.6° (23, DMF)	$C_{16}H_{29}N_3O_6$	53.72 (53.46)	8.10 8.13	11.44 11.69)
4i	L-Ser(OBzl)	$CH_2C_6H_5$	98.0	65—66[c]	+5.7° (23, EtOH)	$C_{27}H_{35}N_3O_7$	62.98 (63.14)	6.93 6.87	8.19 8.18)

a) Lit,[11b] mp 137.5—138.5 °C. b) Aib: α-aminoisobutyric acid. c) Lit,[11c] mp 65—66 °C; $[\alpha]_D^{25}$ +5.8°(MeOH).

N-acetylisomuramic acid (**6b**) by that of Petit and Sinäy.[13] Compound **6c** was obtained by treating benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[12] with sodium hydride, followed by condensation with (*S*)-2-chloro-4-methylpentanoic acid.[14] (The compound obtained after hydrogenolytic deprotection of **6c** is referred to as *N*-acetyl *C*-isobutylnormuramic acid in this text). Acylation of benzyl 2-amino-4,6-*O*-benzylidene-α-D-glucopyranoside[15] with benzyloxyacetic anhydride gave benzyl 4,6-*O*-benzylidene-2-benzyloxyacetamido-α-D-glucopyranoside (**10**), which was then treated with sodium hydride and condensed with (*S*)-2-chloropropionic acid[16] as in the synthesis of **6a** to give protected *N*-glycolylmuramic acid (**6d**). The protected glycodipeptides (**8**) obtained are given in Table 2 together with their physicochemical properties.

The *N*-acetylmuramyl dipeptides (**9a**—**9h**), *N*-acetylisomuramyl dipeptide (**9i**), *N*-glycolylmuramyl dipeptides (**9j**—**9m**) and *N*-acetyl *C*-isobutylnormuramyl dipeptide (**9n**) were obtained after elimination of all the protecting groups by catalytic hydrogenation in acetic acid. The final products (**9**) were purified by column chromatography on Sephadex LH-20 with ethanol–0.1 M acetic acid as a solvent. The physicochemical properties of the desired MDP analogs are given in Table 3.

During the hydrogenolytic deprotection of the isomuramyl derivative (**8i**) in acetic acid, TLC showed the generation of L-alanyl-D-isoglutamine (**13**), an unknown by-product (**11**) and a small amount of isomuramic acid (**12**) as well as the desired *N*-acetylisomuramyl-L-alanyl-D-isoglutamine (**9i**). Attempts to isolate the unknown by-product were unsuccessful, the only compound obtained being isomuramic acid.

Treatment of **9i** in acetic acid at 80 °C for 1 h gave nearly the same result on TLC as that obtained by the hydrogenolytic deprotection of **8i**. Quantitative analysis of the liberated L-alanyl-D-isoglutamine by means of an amino acid analyzer showed that the conversion of **9i** into **11** and **12** reaches up to 49.9%. However, hydrogenolysis of **8i** under aprotic conditions (in DMF) gave **9i** with no side reaction.

The results can be explained by assuming that the deprotected *N*-acetylisomuramyl dipeptide (**9i**) is gradually decomposed to give the intramolecular ester (**11**) and the dipeptide through *N* to *O* migration of the amide bond under acidic conditions. The resulting intramolecular ester can readily be hydrolyzed to isomuramic acid (**12**) (Scheme 2). MDP treated in a similar way gave the dipeptide in only 5% yield, suggesting that the *S*-isomer (**9i**) more easily undergoes migration than its *R*-isomer (**9a**).

Biological Results

The adjuvant activity of the synthetic MDP analogs (**9**) was assayed for the induction of delayed-type hypersensitivity to *N*-acetyl-3-(4-arsonophenylazo)-L-tyrosine (ABA–Tyr) in guinea pigs[17] (Table 4).

We see that the *N*-acetylisomuramyl derivative (**9i**) greatly reduces the adjuvant activity (compare **9a** and **9i**), indicating that the configuration of the lactic acid moiety of MDP has to be the *R*-form to elicit the activity.

Replacement of alanine by β-alanine (**9b**) led to complete loss of activity. This indicates that lengthening of the peptide chain by one methylene group (as in **9b**) is unfavorable, since glycine analog of MDP has a distinct although weak as compared with MDP,

Table 2. Yields and physicochemical properties of the protected glycodipeptides **8**

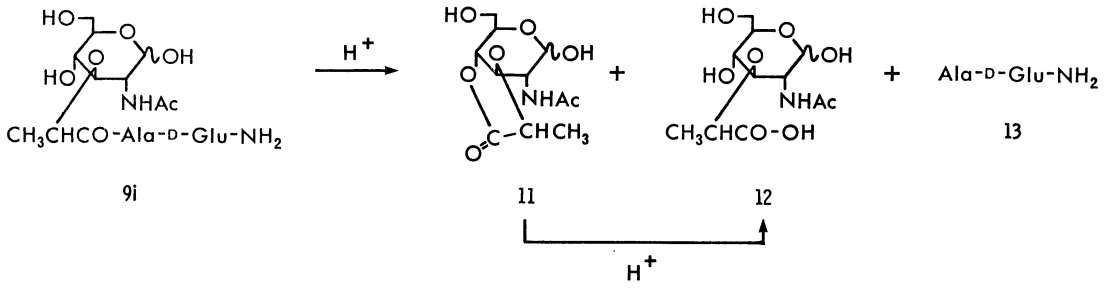
Compd	R_1	R_2	Y	R	Yield/%	Mp/°C	$[\alpha]_D$[a] (c, temp)	Formula	Found(Calcd)[%] C	H	N
8a	CH_3CO	CH_3	L-Ala	Bzl	96.1	223—230 (d)[b]	+89.2° (1.0, 20)	$C_{40}H_{48}N_4O_{11}$	62.91 (63.15	6.12 6.36	7.25 7.40)
8b	CH_3CO	CH_3	β-Ala	Bzl	78.0	276 (d)	+89.9° (0.5, 20)	$C_{40}H_{48}N_4O_{11}$	62.97 (63.15	6.39 6.36	7.27 7.40)
8c	CH_3CO	CH_3	Sar	Bzl	71.8	177—178	+90.1° (0.5, 20)	$C_{40}H_{48}N_4O_{11}$	63.10 (63.15	6.41 6.36	7.27 7.40)
8d	CH_3CO	CH_3	Aib[c]	Bzl	86.7	93 (d)	+87.7° (1.0, 21)	$C_{41}H_{50}N_4O_{11}$	63.82 (63.55	6.88 6.51	6.87 7.23)
8e	CH_3CO	CH_3	L-Pro	Bzl	65.9	82 (d)	+96.4° (0.5, 27)	$C_{42}H_{50}N_4O_{11}$	63.84 (64.11	6.44 6.41	7.07 7.12)
8f	CH_3CO	CH_3	L-Thr(OBzl)	Bzl	65.4	242 (d)	+88.0° (0.1, 27)	$C_{48}H_{56}N_4O_{12}$	65.13 (65.45	6.33 6.41	6.22 6.35)
8g	CH_3CO	CH_3	L-Val	Bzl	93.7	257 (d)	+90.5° (0.5, 20)	$C_{42}H_{52}N_4O_{11}$	63.51 (63.94	6.63 6.64	6.98 7.10)
8h	CH_3CO	CH_3	L-Val	CH_3	92.0	242 (d)	+86.1° (0.5, 22)	$C_{36}H_{48}N_4O_{11}\cdot H_2O$	59.35 (59.16	6.80 6.90	7.70 7.68)
8i[d]	CH_3CO	CH_3	L-Ala	Bzl	58.3	280—281 (d)	+58.3° (0.5, 20)	$C_{40}H_{48}N_4O_{11}\cdot \frac{1}{2}H_2O$	62.56 (62.40	6.50 6.42	7.22 7.28)
8j	$BzlOCH_2CO$	CH_3	L-Ala	Bzl	58.6	196—198	+70.4° (0.4, 27)	$C_{47}H_{54}N_4O_{12}$	64.98 (65.11	6.27 6.28	6.44 6.46)
8k	$BzlOCH_2CO$	CH_3	L-Ser(OBzl)	Bzl	68.0	216—217	+60.0° (0.4, 27)	$C_{54}H_{60}N_4O_{13}$	66.65 (66.65	6.16 6.22	5.67 5.76)
8l	$BzlOCH_2CO$	CH_3	L-Val	Bzl	75.3	199—201	+62.3° (0.4, 27)	$C_{49}H_{58}N_4O_{12}$	65.31 (65.75	6.51 6.53	6.16 6.26)
8m	$BzlOCH_2CO$	CH_3	L-Leu	Bzl	70.2	175—176	+63.2° (0.4, 27)	$C_{50}H_{60}N_4O_{12}$	65.77 (66.06	6.60 6.65	6.00 6.16)
8n	CH_3CO	CH_3>$CHCH_2$ CH_3	L-Ala	Bzl	69.3	234—235	+91.7° (0.5, 23)	$C_{53}H_{54}N_4O_{11}$	64.36 (64.32	6.76 6.78	6.99 6.99)

a) Solvent: DMF. b) Lit,[11b] mp 240 °C(d). c) Aib: α-aminoisobutyric acid. d) The chiral carbon of the lactic acid moiety of this compound has the *S*-configuration.

Table 3. Yields and physicochemical properties of MDP analogs **9**

Compd	R_1	R_2	Y	R	Yield/%	$[\alpha]_D$ (c 0.5) (temp, solvent)	Formula	Found(Calcd)[%] C	H	N
9a	CH_3CO	CH_3	L-Ala	H	85.4	+33.7°(23, H_2O)[a,b,c]	$C_{19}H_{32}N_4O_{11}\cdot\frac{1}{4}H_2O$	45.70 (45.92	6.90 6.59	11.21 11.27)
9b	CH_3CO	CH_3	β-Ala	H	95.5	+31.8°(19, H_2O)[a]	$C_{19}H_{32}N_4O_{11}\cdot H_2O$	44.64 (44.69	6.73 6.71	10.63 10.98)
9c	CH_3CO	CH_3	Sar	H	95.8	+47.7°(19, H_2O)[a]	$C_{19}H_{32}N_4O_{11}\cdot\frac{3}{2}H_2O$	43.70 (43.92	6.91 6.79	10.35 10.78)
9d	CH_3CO	CH_3	Aib[d]	H	quant	+46.3°(19, H_2O)[a,c]	$C_{20}H_{34}N_4O_{11}\cdot\frac{1}{2}H_2O$	46.58 (46.59	6.80 6.84	10.76 10.86)
9e	CH_3CO	CH_3	L-Pro	H	96.7	+60.2°(27, EtOH)	$C_{21}H_{34}N_4O_{11}\cdot\frac{1}{2}H_2O$	47.91 (47.81	7.01 6.69	10.48 10.62)
9f	CH_3CO	CH_3	L-Thr	H	66.4	+51.3°(27, EtOH)[e]	$C_{20}H_{34}N_4O_{12}\cdot H_2O$	45.49 (45.19	6.93 6.64	10.52 10.54)
9g	CH_3CO	CH_3	L-Val	H	94.2	+61.8°(23, DMF)	$C_{21}H_{36}N_4O_{11}\cdot\frac{1}{2}H_2O$	47.98 (47.63	7.41 7.04	10.32 10.58)
9h	CH_3CO	CH_3	L-Val	CH_3	86.1	+53.8°(22, DMF)	$C_{22}H_{38}N_4O_{11}\cdot\frac{1}{2}H_2O\cdot\frac{1}{2}$AcOH	48.34 (48.15	7.22 7.21	9.78 9.77)
9i[f]	CH_3CO	CH_3	L-Ala	H	89.3	−19.1°(24, H_2O)[a]	$C_{19}H_{32}N_4O_{11}\cdot 2H_2O$	43.58 (43.17	6.63 6.87	10.51 10.60)
9j	$HOCH_2CO$	CH_3	L-Ala	H	88.1	+58.1°(27, DMF)	$C_{19}H_{32}N_4O_{12}\cdot\frac{1}{2}H_2O\frac{1}{2}$AcOH	43.98 (43.87	6.88 6.45	10.25 10.23)
9k	$HOCH_2CO$	CH_3	L-Ser	H	86.7	+57.4°(27, DMF)	$C_{19}H_{32}N_4O_{13}\cdot H_2O$	42.12 (42.06	6.70 6.32	9.95 10.32)
9l	$HOCH_2CO$	CH_3	L-Val	H	90.7	+36.1°(27, DMF)	$C_{21}H_{36}N_4O_{12}\cdot H_2O\cdot\frac{1}{2}$AcOH	45.40 (45.20	7.13 6.72	9.54 9.58)
9m	$HOCH_2CO$	CH_3	L-Leu	H	97.1	+51.4°(27, DMF)	$C_{22}H_{38}N_4O_{12}\cdot\frac{1}{2}H_2O$	47.21 (47.23	7.38 7.03	9.66 10.01)
9n	$HOCH_2CO$	$CH_3\diagdown$ $\diagup CH_3$ CHCH$_2$	L-Ala	H	84.8	+39.7°(23, H_2O)[a]	$C_{22}H_{33}N_4O_{11}\cdot\frac{3}{2}H_2O$	47.29 (47.05	7.18 7.36	9.68 9.98)

a) Optical rotation was determined when mutarotation was completed (25 h). b) c 0.1. c) Lit,[11b] $[\alpha]_D^{15}$ +33.1° (after 25 h) (c 0.51, H_2O). d) Aib: α-aminoisobutyric acid. e) c 0.4. f) The chiral carbon of the lactic acid moiety of this compound has the *S*-configuration.



Scheme 2.

TABLE 4. ADJUVANT ACTIVITY OF MDP ANALOGS **9** IN DELAYED-TYPE HYPERSENSITIVITY TO ABA-Tyr (100 μg) IN GUINEA PIGS

Compd[a]	Skin reaction with BαA at 24 h (mm±SE)			
	Exp 1	Exp 2	Exp 3	Exp 4
MurNAc-L-Ala-D-Glu-NH_2 (**9a**)	18.1±1.2	22.9±1.2	18.2±0.7	17.6±0.7
MurNAc-β-Ala-D-Glu-NH_2 (**9b**)	0	—	—	—
MurNAc-Sar-D-Glu-NH_2 (**9c**)	0	—	—	—
MurNAc-Aib-D-Gul-NH_2[b] (**9d**)	21.8±1.7	—	—	—
MurNAc-L-Pro-D-Glu-NH_2 (**9e**)	—	14.1±0.5	—	—
MurNAc-L-Thr-D-Glu-NH_2 (**9f**)	—	24.0±1.1	—	—
MurNAc-L-Val-D-Glu-NH_2 (**9g**)	—	28.0±1.5	20.0±1.0	19.4±0.7
OCH_3 | MurNAc-L-Val-D-Glu-NH_2 (**9h**)	—	—	—	19.7±0.4
isoMurNAc-L-Ala-D-Glu-NH_2 (**9i**)	—	9.4±1.0	—	—
MurNGl-L-Ala-D-Glu-NH_2[c] (**9j**)	—	—	18.0±0.7	—
MurNGl-L-Ser-D-Glu-NH_2[c] (**9k**)	—	—	19.3±0.5	—
MurNGl-L-Val-D-Glu-NH_2[c] (**9l**)	—	—	19.6±0.9	—
MurNGl-L-Leu-D-Glu-NH_2[c] (**9m**)	—	—	16.4±0.5	—
C-IsobutylnorMurNAc-L-Ala-D-Glu-NH_2 (**9n**)	17.5±1.2	—	—	—
Control (ABA-Tyr+FIA)[d]	0	0	0	0

a) Dose: 100 μg. b) Aib: α-aminoisobutyric acid. c) MurNGl=*N*-glycolylmuramyl. d) FIA: Freund's incomplete adjuvant.

adjuvant activity.[18] No activity of the sarcosine analog (**9c**) and reduced activity of the proline analog (**9e**) show the necessity of the NH group adjacent to the lactic acid moiety.

The results indicate that alanine or some other α-amino acid adjacent to the lactic acid moiety is important for high biological activity. Replacement of alanine by α-aminoisobutyric acid (**9e**), threonine (**9f**) and valine (**9g**) gave rise to an increase in activity, the valine analog showing the most favorable effect on biological potency. The *N*-glycolyl leucine analog (**9m**), however, has moderate but slightly weak activity as compared with other *N*-glycolyl analogs (**9j**—**9l**), indicating that the size or lipophilicity of the amino acid adjacent to the lactic acid moiety has an effect on the activity. An increase in the size or lipophilicity of the lactic acid residue in muramic acid (**9n**) also caused a decrease in the activity.

The *N*-glycolylmuramyl analogs have activities equipotent to those of the corresponding *N*-acetylmuramyl analogs (compare **9a** with **9j**, and **9g** with **9l**), indicating that *N*-glycolyl and *N*-acetyl groups caused no marked difference in activity.[19] The methyl ester (**9h**) of the valine analog in which a carboxyl group of the D-isoglutamine residue is esterified also possesses activity equipotent to that of the carboxyl-free analog (**9g**).

Experimental

Melting points were taken in open capillaries and are uncorrected. Optical rotations were determined with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed on a Hitachi KLA-3B amino acid analyzer. All chemicals and solvents were of reagent grade and used without further purification with the exception of dioxane which was dried by refluxing in the presence of CaH_2. The reactions were monitored on TLC with Merck F_{254} silica gel plates, which were developed with $CHCl_3$–MeOH (19:1, v/v) for compounds **4**, $CHCl_3$–acetone–MeOH (10:3:2, v/v) for **8**, and *n*-BuOH–EtOAc–AcOH–H_2O (1:1:1:1, v/v) for **9**. Evaporation was carried out in a rotary vacuum evaporator under reduced pressure at temperatures below 45 °C.

D-*Glutamic Acid γ-Benzyl Ester.* This was synthesized according to the method of Guttman and Boissonnas:[20] mp 176—178 °C (lit for L-form 189 °C,[20] 174 °C[21]); $[\alpha]_D^{25}$ −18.6° (*c* 6.0, AcOH) [lit for L-form[20] $[\alpha]_D^{23}$ +19.6°±0.2° (*c* 6.0, AcOH)].

t-*Butoxycarbonyl*-D-*glutamic Acid γ-Benzyl Ester DCHA Salt.* A suspension of D-glutamic acid γ-benzyl ester (5.93 g, 25

mmol) in water (14 ml) was combined with a DMF solution (14 ml) of *O*-*t*-butyl *S*-(4,6-dimethyl-2-pyrimidinyl)thiocarbonate[22] (7.21 g, 30 mmol), Et_3N (3.5 ml, 25 mmol) being then added. The mixture was stirred overnight, becoming a clear solution, to which was added water (40 ml). After extraction with EtOAc (50 ml), the pH of the aqueous layer was adjusted to 2 by addition of 5 M HCl under cooling and the mixture was extracted with EtOAc (40 ml×3). The EtOAc layer was washed with 1 M HCl (25 ml×3) and satd. aq NaCl (25 ml×2), and dried over Na_2SO_4. Evaporation of the solvent gave an oil (7.00 g) of *t*-butoxycarbonyl-D-glutamic acid γ-benzyl ester, which was converted into its DCHA salt in a mixture of EtOAc (50 ml) and Et_2O (50 ml) by addition of a petroleum ether solution (10 ml) of DCHA (3.63 g, 20 mmol) under cooling. The precipitate was filtered to give crystalline *t*-butoxycarbonyl-D-glutamic acid γ-benzyl ester DCHA salt: 10.15 g (78.3%). Mp 141—143 °C (lit for L-form[23] 138—139 °C). $[\alpha]_D^{19}$ −12.5° (*c* 1.0, MeOH) [lit for L-form[23] $[\alpha]_D^{25}$ +11.9° (MeOH)]. Found: C, 67.01; H, 9.16; N, 5.56%. Calcd for $C_{29}H_{46}O_6N_2$: C, 67.15; H, 8.94; N, 5.56%.

t-*Butoxycarbonyl*-D-*glutamic Acid* α-p-*Nitrophenyl* γ-*Benzyl Diester.* A suspension of *t*-butoxycarbonyl-D-glutamic acid γ-benzyl ester DCHA salt (9.86 g, 19 mmol) in EtOAc (100 ml) was shaken with 0.2 M H_2SO_4 (100 ml). The EtOAc layer was washed with satd. aq NaCl (25 ml×2) and dried over Na_2SO_4. After filtration, *p*-nitrophenol (2.69 g, 19 mmol) and DCC (3.92 g, 19 mmol) were added under cooling, and the mixture was stirred at 0 °C for 2 h and kept in a refrigerator overnight. The resulting *N,N'*-dicyclohexylurea was filtered and the filter cake was washed with EtOAc. The combined EtOAc layer was then concentrated to 50 ml and the resulting precipitate was filtered: 7.55 g (86.8%). Mp 121—122 °C (lit for L-form[24] 120—121 °C). $[\alpha]_D^{23}$ +25.0° (*c* 0.5, EtOAc) [lit for L-form[24] $[\alpha]_D^{25}$ −32.7° (MeOH)]. Found: C, 60.19; H, 5.76; N, 6.07%. Calcd for $C_{23}H_{26}O_8N_2$: C, 60.25; H, 5.72; N, 6.11%.

t-*Butoxycarbonyl*-D-*isoglutamine Benzyl Ester.* *t*-Butoxycarbonyl-D-glutamic acid α-*p*-nitrophenyl γ-benzyl diester (4.58 g, 10 mmol) was dissolved in tetrahydrofuran (40 ml), ammonia water (25%, 6.8 ml) being added to the solution. After being stirred for 2 h at room temperature, the solution was concentrated and the residue was dissolved in EtOAc (150 ml). After the usual work-up, the residue was recrystallized from EtOAc–petroleum ether: 2.38 g (70.8%). mp 123—124 °C. $[\alpha]_D^{20}$ −28.0° (*c* 0.5, MeOH). Found: C, 60.63; H. 7.15; N, 8.07%. Calcd for $C_{17}H_{24}O_5N_2$: C, 60.70; H, 7.19; N, 8.23%.

t-*Butoxycarbonyl*-L-*valine HONB Ester.* *t*-Butoxycarbonyl-L-valine (15.2 g, 70 mmol), HONB (13.8 g, 77 mmol) and DCC (15.9 g, 77 mmol) were combined in a mixture of acetonitrile (300 ml) and DMF under cooling. The mixture was stirred at 0 °C for 1 h and at room temperature for 4 h and filtered. After evaporation of the solvent, the residue was dissolved in EtOAc. The EtOAc layer was washed with 5% $NaHCO_3$ and water, then dried over Na_2SO_4. The solvent was removed and the residue was triturated with petroleum ether giving crystalline *t*-butoxycarbonyl-L-valine HONB ester: 26.5 g (quantitative). mp 120—122 °C. $[\alpha]_D^{27}$ −33.6° (*c* 0.5, DMF). Found: C, 60.14; H, 7.21; N, 7.11%. Calcd for $C_{19}H_{26}O_2N_6$: C, 60.30; H, 6.93; N, 7.40%.

t-*Butoxycarbonyl*-D-*glutamic Acid* γ-*Methyl Ester.* This was synthesized from D-glutamic acid γ-methyl ester[25] (19.7 g, 0.1 mol) in a similar way to that described for *t*-butoxycarbonyl-D-glutamic acid γ-benzyl ester: 23.5 g (89.9%). An analytical sample was converted into its DCHA salt as described for the preparation of *t*-butoxycarbonyl-D-glutamic acid γ-benzyl ester DCHA salt: mp 155 °C (lit for L-form[26] 155—157 °C). $[\alpha]_D^{23}$ −10.2° (*c* 0.5, MeOH) [lit for L-form[26] $[\alpha]_D$ +10.5° (MeOH)]. Found: C, 62.45; H, 9.61; N, 6.17%. Calcd for $C_{23}H_{42}O_6N_2$: C, 62.47; H, 9.57; N, 6.33%.

t-*Butoxycarbonyl*-D-*glutamic Acid* α-N-*Succinimidyl* γ-*Methyl Diester.* To a solution of *t*-butoxycarbonyl-D-glutamic acid γ-methyl ester (23.0 g, 88 mmol) and *N*-hydroxysuccinimide (11.2 g, 97 mmol) in acetonitrile (200 ml) was added DCC (20.0 g, 97 mmol) under cooling, and the mixture was stirred at 0 °C for 3 h and at room temperature for 12 h. After filtration and evaporation of the solvent, the residue was dissolved in EtOAc (175 ml). The EtOAc layer was washed with 5% $NaHCO_3$ (70 ml×3), water (70 ml×3) and 10% citric acid (70 ml×3) successively, and dried over Na_2SO_4. After evaporation, the residue was triturated with petroleum ether giving the desired compound: 22.9 g (72.7%). Mp 96 °C. $[\alpha]_D^{23}$ +33.8° (*c* 0.5, DMF).

t-*Butoxycarbonyl*-D-*isoglutamine Methyl Ester.* *t*-Butoxycarbonyl-D-glutamic acid α-*N*-succinimidyl γ-methyl diester (17.9 g, 50 mmol) was dissolved in tetrahydrofuran (50 ml), ammonia water (25%, 18 ml) being added to the resulting solution. After being stirred at 0 °C for 30 min and at room temperature for 30 min, the solution was concentrated and the residue was dissolved in EtOAc (250 ml). After the usual work-up, the residue was recrystallized from EtOAc–petroleum ether: 6.4 g (49.2%). Mp 129—130 °C. $[\alpha]_D^{23}$ −4.2° (*c* 0.5, DMF). Found: C, 51.08; H, 6.25; N, 7.74%. Calcd for $C_{11}H_{20}O_2N_5$: C, 50.75; H, 6.19; N, 7.82%.

t-*Butoxycarbonyl*-L-*valyl*-D-*isoglutamine Methyl Ester* (***4h***). *t*-Butoxycarbonyl-D-isoglutamine methyl ester (5.0 g, 19.1 mmol) was dissolved in cold trifluoroacetic acid (20 ml) and the resulting solution was stirred at room temperature for 15 min. Trifluoroacetic acid was then removed and the residue was triturated with Et_2O. Oily D-isoglutamine methyl ester trifluoroacetate was dried over NaOH pellets, dissolved in acetonitrile (40 ml), and neutralized with Et_3N (2.8 ml, 20 mmol). *t*-Butoxycarbonyl-L-valine HONB ester (7.22 g, 19.1 mmol) was added to this solution and the mixture was stirred at room temperature for 15 h. The solution was then concentrated and the residue extracted with EtOAc (500 ml). The EtOAc layer was washed successively with 5% $NaHCO_3$, 10% citric acid, and water (100 ml×3), then dried over Na_2SO_4 and evaporated. The residue was triturated with petroleum ether giving crystals which were recrystallized from EtOAc–petroleum ether: 5.8 g (84.3%).

Other protected dipeptides (***4***) were similarly prepared from appropriate *t*-butoxycarbonylamino acid HONB esters and *t*-butoxycarbonyl-D-isoglutamine benzyl ester.

*Benzyl 4,6-*O*-Benzylidene-2-benzyloxyacetamido-2-deoxy-*α*-*D*-glucopyranoside* (***10***). DCC (7.48 g, 36.3 mmol) was added at 0 °C to a solution of benzyloxyacetic acid[27] (11.0 g, 66 mmol) in dichloromethane (100 ml). The mixture was stirred at 0 °C for 2 h and at room temperature for 15 h. After filtration, the solvent was removed to give oily bis(benzyloxyacetic) anhydride which was dissolved in tetrahydrofuran (300 ml) without further purification together with benzyl 2-amino-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside[15] (10.7 g, 30 mmol). The mixture was heated under reflux for 1 h and the solvent was evaporated. The resulting crystalline residue was collected by filtration and washed with Et_2O to give **10** in a quantitative yield: 15.2 g. Mp 161—162 °C. $[\alpha]_D^{27}$ +48.6° (*c* 0.5, $CHCl_3$). Found: C, 68.70; H, 6.31; N, 2.40%. Calcd for $C_{29}H_{31}ON_7$: C, 68.89; H, 6.18; N, 2.77%.

*Benzyl 2-Acetamido-4,6-O-benzylidene-3-O-[1-(R)-carboxyisopentyl)-2-deoxy-α-D-glucopyranoside (**6c**).* Benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (3.2 g, 8 mmol) was dissolved in dioxane (300 ml) at 90 °C. Sodium hydride (1.80 g, 60% in oil) was then added to the solution at 70 °C and the mixture was kept at 90 °C for 1 h. A solution of (*S*)-2-chloro-4-methylpentanoic acid[14] (6.3 g, 42 mmol) in dioxane (10 ml) was added when the temperature had cooled to 65 °C. After 1 h, additional sodium hydride (7.48 g) was added and the mixture was stirred at 65 °C for 15 h. Water was then added carefully to the reaction mixture under cooling, and the dioxane of the upper phase separated by decantation was evaporated. The residue was dissolved in water (100 ml) and the solution extracted with $CHCl_3$ (50 ml). The aqueous phase was adjusted to pH 3 with 2 M HCl under cooling, the precipitate being extracted with $CHCl_3$ (100 ml×3). The combined organic layer was washed with water and dried (Na_2SO_4). After filtration and evaporation, the residue was purified by silica-gel column (4×12 cm) chromatography using $CHCl_3$–MeOH (95:5, v/v) as solvent: 1.2 g (29.9%). Mp 156—157 °C. $[\alpha]_D^{23}$ +118.4° (*c* 0.5, DMF). Found: C, 65.56; H, 6.88; N, 2.74%. Calcd for $C_{27}H_{36}ON_8$: C, 65.48; H, 6.87; N, 2.73%.

*Benzyl 4,6-O-Benzylidene-2-benzyloxyacetamido-3-O-[1-(R)-carboxyethyl]2-deoxy-α-D-glucopyranoside (**6d**).* Sodium hydride (2.0 g, 50 mmol, *ca.* 60% in oil) was added to a dioxane (300 ml) solution of **10** (10.1 g, 20 mmol) and the mixture was stirred at 90 °C for 1 h. (*S*)-2-Chloropropionic acid (7.54 g, 70 mmol) was added when the temperature had cooled to 65 °C. After 1 h, additional sodium hydride (3.2 g, 80 mmol, 60% in oil) was added and the mixture was stirred at 65 °C for 15 h. Water (40 ml) was then added carefully under cooling, the pH of mixture being adjusted to 7 with 10% citric acid. After evaporation of dioxane, 10% citric acid (250 ml) was added. The resulting precipitate was filtered and washed with water, then with $CHCl_3$–petroleum ether. Recrystallization from MeOH gave **6d**: 6.70 g (58.1%). Mp 230 °C (dec.). $[\alpha]_D^{27}$ +67.5° (*c* 0.3, DMF). Found: C, 65.59; H, 5.89; N, 2.50%. Calcd for $C_{32}H_{35}ON_9 \cdot {}^1/_2H_2O$: C, 65.51; H, 6.19; N, 2.38%.

*Benzyl 2-Acetamido-4,6-O-benzylidene-3-O-[1-(R)-carboxyethyl]-2-deoxy-α-D-glucopyranoside HONB Ester (**7a**).* DCC (3.71 g, 18 mmol) was added to a solution of benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[1-(*R*)-carboxyethyl]-2-deoxy-α-D-glucopyranoside[12] (7.07 g, 15 mmol) and HONB (3.22 g, 18 mmol) in tetrahydrofuran (110 ml) under cooling, and the mixture was stirred at 4 °C for 3 h and at room temperature for 16 h. After filtration, the solvent was removed and the resulting crystalline residue was recrystallized from EtOAc–Et_2O: 7.42 g (78.4%). Mp 122—124 °C. $[\alpha]_D^{27}$ +86.0° (*c* 0.5, DMF). Found: C, 64.52; H, 5.67; N, 4.25%. Calcd for $C_{34}H_{36}O_{10}N_2$: C, 64.55; H, 5.74; N, 4.43%.

*Benzyl 4,6-O-Benzylidene-2-benzyloxyacetamido-3-O-[1-(R)-carboxyethyl)-2-deoxy-α-D-glucopyranoside HONB Ester (**7d**)* was synthesized in a similar way to that described for **7a**: 99.6%. Mp 98—99 °C. $[\alpha]_D^{27}$ +69.0° (*c* 0.5, DMF). Found: C, 66.64; H, 5.99; N, 3.77%. Calcd for $C_{41}H_{42}O_{11}N_2$: C, 66.65; H, 5.73; N, 3.79%.

*N-Acetyl-1-O-Benzyl-4,6-O-benzylidene-α-muramyl-L-valyl-D-isoglutamine Methyl Ester (**8h**).* *t*-Butoxycarbonyl-L-valyl-D-isoglutamine methyl ester (**4h**) (359 mg, 1 mmol) was treated with trifluoroacetic acid (5 ml) at room temperature for 20 min. After evaporation, the residue was triturated with Et_2O and collected. L-Valyl-D-isoglutamine methyl ester trifluoroacetate thus obtained was dissolved in acetonitrile (10 ml) and the solution was neutralized with Et_3N (0.17 ml) under cooling. A solution of **7a** (633 mg, 1 mmol) in acetonitrile (10 ml) was added and the the mixture was stirred at room temperature for 15 h. The resulting precipitate was collected and washed with hot acetonitrile to give **8h**: 656 mg (92.0%).

*Other protected muramyl dipeptides (**8**)* were similarly prepared from appropriate protected muramic acid HONB esters and protected dipeptides.

*N-Acetylmuramyl-L-valyl-D-isoglutamine Methyl Ester (**9h**).* Compound **8h** (31.2 mg, 0.05 mmol) was hydrogenolyzed in a mixture of MeOH (5 ml) and AcOH (0.5 ml) with palladium black as a catalyst at room temperature for 3 h. After filtration and evaporation, the residue was kept in a refrigerator to allow crystallization. Et_2O was then added and the crystals were filtered to give **9h**: 23 mg (86.1%).

*Other muramyl dipeptides (**9**)* were similarly prepared from appropriate protected muramyl dipeptides (**8**).

*Isolation of N-Acetylisomuramic Acid (**12**) and L-Alanyl-D-isoglutamine (**13**) from a Side Reaction.* *N*-Acetyl-1-*O*-benzyl-4,6-*O*-benzylidene-α-isomuramyl-L-alanyl-D-isoglutamine benzyl ester (**8i** 857 mg) was subsjected to hydrogenolysis in AcOH (50 ml) in the presence of palladium black at room temperature for 5 d. After filtration and evaporation, the residue was applied to a column of Sephadex LH-20 (3×90 cm) and eluted with 0.1 M AcOH. The fractions from 155 to 175 ml containing the probable internal ester (**11**) and *N*-acetylisomuramic acid (**12**), identical on TLC with an authentic sample,[13] were collected and lyophilized. The residual syrup was solidified with Et_2O and filtered. The powder obtained was highly hygroscopic, showing a single spot corresponding to *N*-acetylisomuramic acid on TLC: 60 mg. Found: C, 42.25; H, 6.50; N, 4.43%. Calcd for $C_{11}H_{19}ON_8$: C, 42.44; H, 6.78; N, 4.50%.

Other 125—140 ml fractions were also collected and lyophilized. The resulting powder was applied to a column (2×40 cm) of CM-cellulose and eluted with dilute AcOH (pH 4.5). The fraction 63—98 ml was collected and lyophilized to give L-alanyl-D-isoglutamine (**13**, 95 mg), which had an R_f value on TLC identical to that of the authentic dipeptide. Amino acid analysis (hydrolysis with 6 M HCl at 110 °C for 24 h): Ala, 1.00; Glu, 1.01.

*Comparison of Degradation (Side Reaction) Rate of **9a** and **9i**.* Solutions of AcOH (0.5 ml each) containing *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**9a**, 10.34 μmol) or *N*-acetylisomuramyl-L-alanyl-D-isoglutamine (**9i**, 10.10 μmol) were heated at 80 °C for 1 h. After evaporation, the residue was subjected to amino acid analysis (column length: 20 cm, buffer: pH 5.28, flow rate: 60 ml/h). The quantity of liberated dipeptide was calculated on the basis of the standard experiment using L-alanyl-D-isoglutamine[11b] (retention time: 25 min), and was 0.52 μmol (5.0%) from **9a** and 5.04 μmol (49.9%) from **9i**.

Biological Assay. Determination of the adjuvant activity on the induction of delayed-type hypersensitivity was carried out according to the method reported.[17]

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References

1) Previous communication in this series: S. Kobayashi, T. Fukuda, I. Imada, M. Fujino, I. Azuma, and Y. Yamamura, *Chem. Pharm. Bull.*, **27**, 3193 (1979).
2) J. Freund, *Adv. Tuber. Res.*, **7**, 130 (1956).
3) F. Ellouz, A. Adam, R. Ciorbaru, and E. Lederer, *Biochem. Biophys. Res. Commun.*, **59**, 1317 (1974).

4) S. Kotani, Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka, *Biken J.*, **18**, 105 (1975).

5) I. Azuma, K. Sugimura, T. Taniyama, M. Yamawaki, Y. Yamamura, S. Kusumoto, S. Okada, and T. Shiba, *Infect. Immun.*, **14**, 18 (1976); F. Audibert, L. Chedid, P. Lefrancier, and J. Choay, *Cellular Immunol.*, **21**, 243 (1976); A. Adam, M. Devys, Vongthip Souvnnavong, P. Lefrancier, and E. Lederer, *Biochem. Biophys. Res. Commun.*, **72**, 339 (1977); L. Chedid, F. Audibert, and A. G. Johnson, *Prog. Allergy*, **25**, 63 (1978).

6) L. Chedid, M. Parant, F. Parant, P. Lefrancier, J. Choay, and E. Lederer, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2089 (1977).

7) C. Damais, M. Parant, and L. Chedid, *Cell Immunol.*, **34**, 49 (1976); C. Damais, M. Parant, L. Chedid, P. Lefrancier, and J. Choay, *ibid.*, **35**, 173 (1977).

8) A. Tanaka, S. Nagao, R. Saito, S. Kotani, S. Kusumoto, and T. Shiba, *Biochem. Biophys. Res. Commun.*, **77**, 621 (1977).

9) Y. Yamamoto, S. Nagao, A. Tanaka, T. Koga, and K. Onoue, *Biochem. Biophys. Res. Commun.*, **80**, 923 (1978).

10) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.*, **22**, 1857 (1974).

11) a) C. Merser, P. Sinäy, and A. Adam, *Biochem. Biophys. Res. Commun.*, **66**, 1316 (1975); b) S. Kusumoto, Y. Tarumi, K. Ikenaka, and T. Shiba, *Bull. Chem. Soc. Jpn.*, **49**, 533 (1976); c) P. Lefrancier, J. Choay, M. Derrien, and I. Lederman, *Int. J. Peptide Protein Res.*, **9**, 249 (1977); d) A. Hasegawa Y. Kaneda, M. Amano, M. Kiso, and I. Azuma, *Agric. Biol. Chem.*, **42**, 2187 (1978).

12) H. M. Flowers and R. W. Jeanloz, *J. Org. Chem.*, **28**, 2983 (1973).

13) J. M. Petit and P. Sinäy, *Carbohydr. Res.*, **24**, 415 (1972).

14) K. Ichimura and M. Ohta, *Bull. Chem. Soc. Jpn.*, **43**, 1443 (1970).

15) P. H. Gross and R. W. Jeanloz, *J. Org. Chem.*, **32**, 2759 (1967).

16) S. C. J. Fu, S. M. Birnbaum, and J. P. Greenstein, *J. Am. Chem. Soc.*, **76**, 6054 (1954).

17) M. Uemiya, K. Sugimura, T. Kusama, I. Saiki, M. Yamawaki, I. Azuma, and Y. Yamamura, *Infect. Immun.*, **24**, 83 (1979).

18) A. Adam, M. Devys, V. Souvannavong, P. Lefrancier, J. Choay, and E. Lederer, *Biochem. Biophys. Res. Commun.*, **72**, 339 (1976).

19) *N*-Glycolylmuramic acid is a constituent of *Nocardiae* and *Mycobacteria*, and *N*-acetylmuramic acid that of *Corynebacteria*. The cell walls of these bacteria cause induction of delayed-type hypersensitivity; for example, see Ref. 18.

20) St. Guttman and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).

21) E. R. Blout and R. H. Karlson, *J. Am. Chem. Soc.*, **78**, 941 (1956).

22) T. Nagasawa, K. Kuroiwa, K. Narita, and Y. Iwasa, *Bull. Chem. Soc. Jpn.*, **46**, 1269 (1973).

23) E. Schröder and E. Klieger, *Ann.*, **673**, 196 (1964).

24) C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).

25) M. Goodman, E. E. Schmitt, and D. A. Yphantis, *J. Am. Chem. Soc.*, **84**, 1283 (1962).

26) J. C. Anderson, M. A. Barton, P. M. Hardy, G. W. Kenner, J. Preston, and R. C. Sheppard, *J. Chem. Soc., C*, **1967**, 108.

27) H. O. L. Fischer and B. Gohlke, *Helv. Chim. Acta*, **16**, 1130 (1933).