Enzymatic Synthesis and Comparative Biological Evaluation of a Phosphonate Analogue of the Lipid A Precursor

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Phosphonate analogue **5** of the lipid A precursor **4** has been prepared from phosphonate **2** and nucleotide **3** with the help of lipid A synthase, isolated from the overproducing *Escherichia coli* mutant MC 1061 (Δ2512) or JB1104 (Δ2514). The biological properties of phosphonate **5** and phosphate **4** are quite similar to each other as compared in the limulus amoebocyte lysate assay, by the activation of the RAW264 murine macrophagelike cell line (determined by stimulation of ornithine decarboxylase), and by the pyrogenicity in rabbits. Hydrolytic removal of the 1-phosphate group of **4** is thus not a prerequisite for its biological activity.

Introduction

The lipid A component of lipopolysaccharides (LPS) is the dominant amphipathic structural element on the outer surface of the outer membrane of Gram-negative bacteria.[1] It is responsible for many of the biological properties of endotoxin.[2-7] Endotoxins, while being powerful immunostimulants, also cause several pathophysiological effects. Therefore, we have initiated a research program to dissect the detrimental and the anticipated beneficial effects of lipid A.[8,9]

Lipid A is a disaccharide consisting of two β-1,6-linked D-glucosamine residues which are N- and O-acylated by fatty acids and carry phosphate groups in positions 4′ and 1.[10] Its structure is highly conserved among different enterobacterial strains. An enzyme, lipid A synthase, has been identified[11,12] which catalyzes the glycosidation of 2,3-diacylglucosamine 1-phosphate (lipid X, **1**) by uridine diphosphate 2,3-diacylglucosamine **3** to generate the lipid A precursor **4**,[10] a β-(1–6) linked tetraacyldisaccharide 1-phosphate intermediate (Scheme I). We have used this enzyme (produced by *Escherichia coli*) for the preparation of new lipid A analogues taking advantage of its broad substrate specificity, which allows the synthesis of analogues, modified at the reducing, the nonreducing, or at both moieties.[8,9]

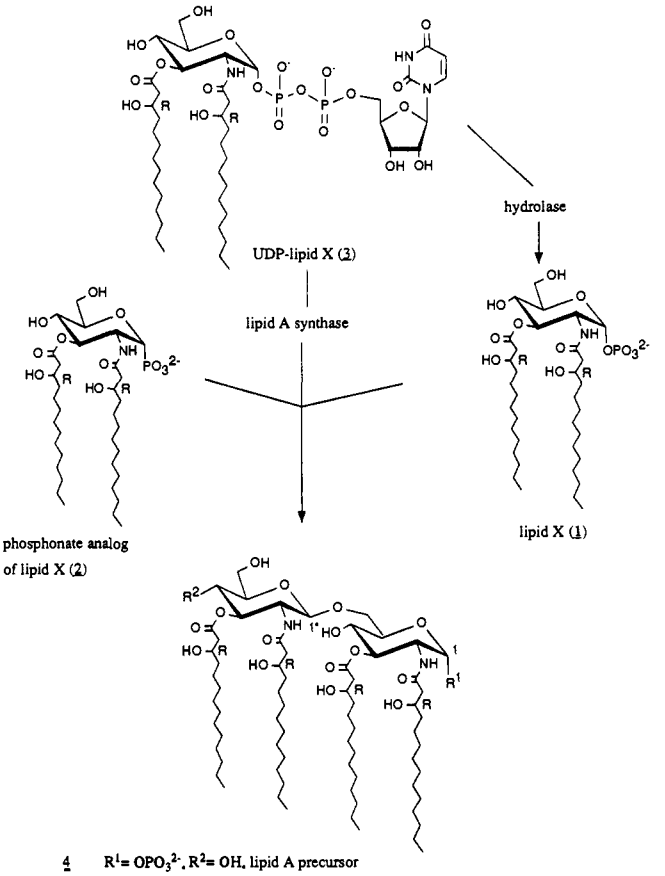
Glycosyl 1-phosphates are rather labile. Hydrolytic cleavage of the phosphate group of lipid A precursor **4** leads to the corresponding hemiacetal. This product is poorly soluble under conditions of biological assays. The phosphate group of **4** may thus be of indirect (solubility, aggregation) or of direct importance for the biological activity. To investigate the relevance of the 1-phosphate group for the biological activity of **4**, we intended to prepare the isopolar, nonisosteric, hydrolytically stable glycosyl-1-phosphonate **5**.[13] At the same time we wished to see if the *E. coli* lipid A synthase accepts the monosaccharide phosphonate **2** as a substrate. Both **2** and the other required substrate UDP-lipid X (**3**) have been synthesized before.[12,14,15] Analogues of lipid A have been obtained by multistep chemical synthesis,[5] which may be considerably simplified by using appropriate enzymes.

To compare the biological properties of **4** and **5**, we chose to study the limulus amoebocyte lysate (LAL) assay[16] for the detection of endotoxic properties in vitro, the ability to enhance ornithine decarboxylase (ODC) activity in the murine macrophagelike cell line RAW264[17,18] as a parameter for macrophage stimulation, and the pyrogenicity test in rabbits.

Chemistry

We used a crude lipid A synthase preparation from an *E. coli* strain (MC 1061/p5r8 Δ2515), overproducing lipid A synthase 1000-fold, constructed by Raetz.[19] This allows

Scheme I



4 $R^1 = OPO_3^{2-}$, $R^2 = OH$, lipid A precursor
5 $R^1 = PO_3^{2-}$, $R^2 = OH$, phosphonate analog of lipid A precursor
6 $R^1 = R^2 = OPO_3^{2-}$, lipid A
7 $R^1 = R^2 = OH$
8 $R^1 = OH$, $R^2 = OPO_3^{2-}$

Table I. Endotoxic Activity of **4**, **5**, and LPS, Determined by LAL Assay

	EU/g
lipid A precursor **4**	8×10^5
phosphonate analogue **5**	3×10^4
LPS	1.2×10^{10}

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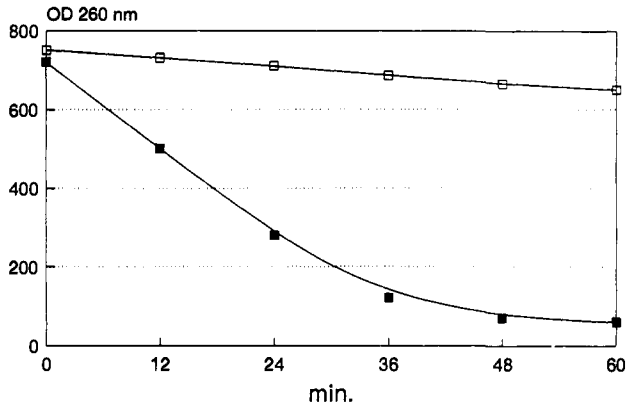

Figure 1. Comparison of the reaction rates for the formation of **4** (solid squares) and **5** (open squares) by spectroscopical monitoring of the consumption of UDP-lipid X (**3**).

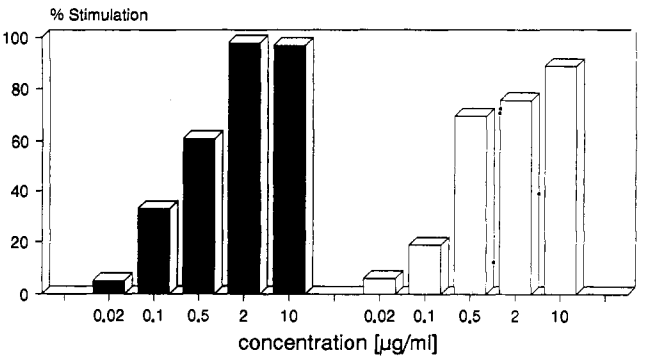

Figure 2. Stimulation of ODC activity in RAW264 cells by **4** (solid bars) and **5** (open bars). Values are expressed as percentage of ODC stimulation obtained with 5 ng/mL LPS. At this concentration of LPS, maximal stimulation of ODC is observed (data not shown).

the synthesis of analogue **5** at an acceptable rate, requiring 3–5 days for the preparation of about 100 mg of crude product. Purification of **5** was difficult due to its poor solubility and contamination with **4**. This contamination is caused by traces of a hydrolase in the crude lipid A synthase preparation, generating lipid X (**1**) from **3**. The rapid reaction of **1** and **3** forms **4** (Scheme I). The method of choice for the purification of **5** was reversed-phase chromatography using a water/pyridine gradient. After purification, the maximal residual contamination with **4** was less than 0.5% based on quantitative high performance thin-layer chromatography. The expected β-D-configura-

Table II. Sum of the Individual Temperature Increases of Three Rabbits following Treatment with **4** and **5**

	dose, μg/kg	increase, °C
4	1	2.1
	50	5.95
5	1	2.1
	50	6.6

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tion of the newly formed glycoside linkage of **5** is evidenced by a value for $J_{1,2}$ of 7.5 Hz.

To determine the relative reaction rate for the formation of **5** as compared to **4**, the consumption of **3** was monitored spectroscopically. The water-soluble uridine phosphate which possesses about the same UV absorption as **3** was removed from periodically withdrawn aliquots of the reaction mixture. For the kinetic experiments, we used a hydrolase-free lipid A synthase preparation obtained from *E. coli* strain JB1104 Δ2514, which is devoid of hydrolase activity.[20] This strain does not overproduce lipid A synthase and is therefore not a suitable enzyme source for the synthesis of **5** on a preparative scale.

As shown in Figure 1, UDP-lipid X (**3**) reacts with its natural substrate lipid X (**1**) approximately 100 times faster than with the phosphonate analogue **2**.

Biology

LPS and lipid A exhibit a wide variety of biological activities, such as pyrogenicity, lethality in mice, the local Shwartzman reaction, induction of nonspecific resistance to infection, polyclonal activation of B-lymphocytes, activation of monocytes and macrophages, and limulus amoebocyte lysate (LAL) clotting.[5,21] The three biological assays we chose to study the effect of substitution of the 1-phosphate group of the lipid A precursor by a 1-phosphonate group represent three different levels of biological complexity, namely a cell-free enzymatic test (LAL), a cellular in vitro assay (RAW264 stimulation), and an in vivo test in animals (pyrogenicity in rabbits).

The biological activity of LPS is higher by several orders of magnitude than that of the lipid A precursor and its analogues. Hence, trace contaminations of endotoxin in all assay systems would significantly falsify all biological data. Consequently, the disaccharides **4** and **5** were purified chromatographically until their specific LAL activity remained at a constant level. We assume that this level reflects the intrinsic endotoxic activity of **4** and **5**. A series of different lipid A precursor analogues has been generated using the same strategy for synthesis and purification as the one described for **4** and **5**.[8] Some of these analogues are almost completely devoid of endotoxinlike activity. This strongly indicates that endotoxin contaminations, if at all present in the crude preparations, are efficiently removed by the described purification strategy. The endotoxic content of all buffer solutions was below 10 pg/mL.

The LAL test is the only assay for the detection of endotoxic activity in vitro.[16] There are various LAL methodologies.[21] For practical reasons, a chromogenic assay in microtiter plates was used.[22] As shown in Table I, lipid A precursor **4** is somewhat more active in this assay than phosphonate analogue **5**. Under the same conditions LPS is about 10^5 times more active than **4** and **5**.

Ornithine decarboxylase (ODC; EC 4.1.1.17) is the first enzyme in the biosynthesis of polyamines, which plays essential roles in cell proliferation and differentiation.[18] ODC activity increases in macrophages within 2 h after LPS addition,[23] the effect being enhanced by priming the macrophage with γ-IFN. Thus, this parameter can be used as a general marker for the activation of macrophages and particularly of RAW264, a macrophage-like tumor cell line of murine origin. We used this parameter to determine the stimulation of the RAW264 cell line by LPS and by **4** and **5**.

As shown in Figure 2, **4** and **5** are active to a similar extent in a concentration-dependent manner. At a concentration of 500 ng/mL both **4** and **5** stimulate RAW 264 cells almost to the same extent as 5 ng/mL LPS, used as internal standard.

The rabbit pyrogen test is the standard assay to measure pyrogenicity. It is based on the postinjection rise of the rabbit's body temperature.[24] As shown in Table II, **4** and **5** are equally effective in this assay.

Discussion

Powerful methods for nonenzymatic glycosidations have been developed. They require sophisticated protecting-group strategies and result in multistep procedures. The use of transglycosylating enzymes for the generation of di- and oligosaccharides from unprotected monosaccharide precursors is therefore an attractive alternative.[25] In this respect, it is encouraging to note the broad substrate specificity of lipid A synthase, which is here documented by its accepting a phosphonate analogue of a natural phosphate.

Both compounds are equally active in an in vitro assay (ODC induction in RAW264 murine macrophage cell line) and in an in vivo test (pyrogenicity in rabbits). In the LAL assay, the specific endotoxic activity of **5** is about 30-fold lower than that of **4**. Considering the well-known problems of reproducibility and the tolerance margin of the LAL test, this difference is relatively small, and its biological significance remains to be evaluated in detail. The similar biological activities of **4** and **5** show that hydrolytic removal of the 1-phosphate group of **4** is not required for its biological activity. This may indicate a specific interaction of this group with a hypothetical receptor—in which case the phosphono group is a good mimic of the phosphate group—or, alternatively, that the 1-phosphate group is only indirectly relevant for the biological activity. Unfortunately, comparable biological data are not available for hemiacetal **7**, corresponding to **4** and **5**, on account of its very poor solubility. In this context, one should note the biological activity and the solubility of 4′-phosphate **8** and of lipid A analogues, carrying for example a 1-phosphoryloxyethyl group.[5d]

The assumption that **4**, **5**, **8**, and closely related analogues interact with the same receptors is plausible. Hence, the 1- and 4′-phosphate groups are either relevant only because they confer a sufficient degree of solubility (which may, in turn, influence upon the degree of aggregation), but not on account of a specific interaction with the receptor, or, alternatively, because the 4′-phosphate group may replace the 1-phosphate group or vice-versa in such an interaction. In any case, both the phosphorylation of other hydroxyl groups and the introduction of other polar groups, ensuring a sufficient degree of solubility, may

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contribute to clarify the role of the phosphate groups.[9]

Experimental Section

^{1}H-NMR spectra were recorded with a Bruker WM-400 instrument (J values are in hertz); ^{31}P-NMR spectra were recorded with ^{1}H broad band decoupling with a Bruker WM-300 instrument. A second ^{1}H-NMR spectrum of **5** was recorded with a Bruker WM-500 instrument at 330 K, shifting the HDO peak to higher fields and allowing to identify the C-1′ proton. Elemental analyses were determined by Dr. J. Zak (Mikroanalytisches Laboratorium, Institut für Physikalische Chemie, Universität Wien). Analytical thin-layer chromatography was performed on high-performance silica gel 60 F_{254} glass plates (HPTLC, Merck). Solvents were AR grade and used without purification. Pyrogen-free water was used throughout. All reagents were obtained from commercial suppliers and were used without further purification. All evaporations were carried out in vacuo with a rotary evaporator.

Preparation of Lipid A Synthase. A 55-L Giovanola 70C fermenter (medium: LB-broth, 1% tryptone, 0.5% yeast extract, 1% NaCl, 1% fructose, 50 mM phosphate buffer (pH 7), 50 mg/L ampicillin, 0.1 ml/L Glanapon 2000) was inoculated at 40 °C with 2% of a culture of *E. coli* MC1061 (Δ2515) or JB1104 (Δ2514) grown overnight in the LB-broth mentioned above. The fermentation was performed with the following parameters: starting OD_{576} = 0.095; stirring, 1000 rmp; air rate, 40 L/min, pO_2 = 40% saturation; temperature, 30 °C. The pH was not regulated and decreased to 6.6. After 3 h of fermentation (OD_{576} = 0.70), 10 g/L fructose and 5 g/L arabinose were added simultaneously.

Subsequently, 270 mL/h of a solution of 187.5 g/L arabinose and 137.5 g/L fructose was added continuously for 12 h (final OD_{576} = 6.40). The cells were harvested by centrifugation (8500g, 10 min), washed, and resuspended in a 10 mM Tris·HCl buffer (pH 7.0) containing 0.2 mM EDTA and 0.2 mM DTE. The cells were disrupted by ultrasonication (~100 W/5 min). After centrifugation (30000g, 4 °C, 20 min) and ultracentrifugation (100000g, 4 °C, 90 min) a fractionated $(NH_4)_2SO_4$ precipitation followed. The pellets of 0–20% and 20–40% $(NH_4)_2SO_4$ saturation were discarded, and the pellet of the 70% $(NH_4)_2SO_4$ saturation was resuspended in buffer as used before. This enzyme preparation was ready to use and was stored at −20 °C.

Enzymatic Preparation of Disaccharides. A 5 mM solution of 2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[[(*R*)-3-hydroxytetradecanoyl]amino]-α-D-glucopyranosyl 1-phosphate (**1**)[14] (20 mL, 71 mg) or of 2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl)-2-[[(*R*)-3-hydroxytetradecanoyl]amino]-α-D-glucopyranosyl-1-phosphonate (**2**)[15] (69.5 mg) in 10 mM Tris.HCl buffer (pH 7.0), 0.2 mM EDTA, and 0.2 mM DTE was mixed with 20 mL of a 5 mM solution of uridine diphosphate 2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[[(*R*)-3-hydroxydecanoyl]amino]-α-D-glucopyranose (**3**)[12] (126 mg) in the same buffer, and 40 mL of the Lipid-A synthase preparation. Triton X-100 was added to a concentration of 0.1%. The mixture was incubated at 30 °C until complete consumption of the UDP derivative (thin-layer control: silica gel, $CHCl_3$/MeOH/CH_3COOH/H_2O = 65/25/5/5; detection spraying with a mixture of 2 g of vanillin, 20 mL of C_2H_5OH, 70 mL of H_2O, and 10 mL of H_2SO_4). The mixture was poured into a flask containing 10 g of prewashed RP18 silica gel (Merck) and 100 mL of 50% aqueous pyridine containing 1% CH_3COOH. The silica gel was washed three times before use with 40% aqueous pyridine to remove excess organosilicon compounds. The flask was shaken for 20 min. After sedimentation of the silica gel the liquid was carefully decanted and, after thin-layer control, discarded. A suspension of the silica gel in 100 mL of 40% aqueous pyridine containing 1% CH_3COOH was slowly poured on top of a column containing 30 g of prewashed RP18 silica gel (Merck). The compound was eluted using a gradient of pyridine/H_2O/CH_3COOH = 70/29/1 to pyridine/H_2O/CH_3COOH = 98/1/1. This procedure was repeated until the specific LAL activity of the desired product remained constant (usually after two runs).

Fractions containing the product were pooled, the solvent was evaporated in vacuum, 50 mL of pyrogen-free H_2O was added, and the solution was lyophilized. The residue was washed twice with ether to remove organosilicon contaminations and dried to yield acid **4** or **5**.

6-*O*-[2-Deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[[(*R*)-3-hydroxytetradecanoyl]amino]-β-D-glucopyranosyl]-2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[[(*R*)-3-hydroxytetradecanoyl]amino]-α-D-glucopyranosyl phosphate, 4: identical [chromatography and ^{1}H-NMR spectroscopy] with an authentic sample obtained by Raetz.[11] Yield: 63%, 83.5 mg R_f = 0.53 (chloroform/methanol/acetic acid/H_2O = 25/15/2/4). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 1/1): δ (vs TMS) 5.49 (br s, H-C(1)), 5.13 (dd, 't', H-C (3)), 4.95 (dd, 't', H-C(3′)), 4.65 (d, H-C(1′)), 4.6–4.45 (br s, HDO), 4.17 (br d, H-C(2)), 4.06–3.98 (m, H-C(5), H_A-C(6)), 4.0–3.95 (m, 2 CH_2CHOH), 3.90–3.85 (m, 2 CH_2CHOH), 3.86 (m, H_A-C(6′)), 3.78 (m, H_B-C(6)), 3.72 (m, H_B-C(6′)), 3.6–3.5 (m, H-C(4), H-C(4′)), 3.36 (H-C(5′)), 2.45 (dd) and 2.35 (dd, 2 $COCH_2$), 2.30 (dd) and 2.20 (dd, 2 $COCH_2$), 1.50–1.35 (m, 8 H, $CHCH_2CH_2$), 1.35–1.20 (br s, 72 H, CH_2), 0.85 (t, 4 CH_3). ^{31}P NMR (300 MHz, pyridine; ^{1}H broad band decoupling): δ (vs phosphoric acid) 3.5 (s). $C_{68}H_{129}N_2O_{20}P$ (1325.7) calcd: C, 61.61; H, 9.81; N, 2.11. Found: C, 60.97; H, 9.43; N, 2.48.

6-*O*-[2-Deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[[(*R*)-3-hydroxytetradecanoyl]amino]-β-D-glucopyranosyl]-2-deoxy-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[[(*R*)-3-hydroxytetradecanoyl]amino]-α-D-glucopyranosyl-phosphonate, 5. Yield: 15%, 19.6 mg. R_f = 0.55 (chloroform/methanol/acetic acid/H_2O = 25/15/2/4). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ = 1/1): δ (vs TMS) 5.63 (dd, $J_{2,3}$ = 10.2, $J_{3,4}$ = 9.1, H-C(3)), 5.05 (dd, $J_{2,3}$ = 10.4, $J_{3,4}$ = 9.1, H-C(3′)), 4.98–4.92 (m), 4.85–4.75 (m), 4.43 (m), 4.26 (ddd, $J_{1,2}$ = 6.9, $J_{2,3}$ = 10.2, $J_{2,P}$ = 25.7, H-C(2)), 4.01 (dd, $J_{1,2}$ = 6.9, $J_{1,P}$ = 11.3, H-C(1)), 3.98–3.85 (m, 4 CH_2CHOC), 3.82–3.70 (m), 3.68–3.62 (m), 3.6–3.5 (m, H-C(4′)), 3.40 (ddd, J = 9.9, 4.8, 2.2), 3.22 (dd, 't', $J_{3,4}$ = 9.1, $J_{4,5}$ = 9.8, H-C(4)), 2.48 (dd, J_{gem} = 15.2, J_{vic} = 4.4) and 2.40 (dd, J_{gem} = 15.2, J_{vic} = 8.4, 2 $COCH_2$), 2.30 (dd, J_{gem} = 14.3, J_{vic} = 3.5) and 2.22 (dd, J_{gem} = 14.3, J_{vic} = 9.0, 2 $COCH_2$), 1.44 (m, 8 H, $CHCH_2CH_2$), 1.26 (br s, 72 H, CH_2), 0.87 (t, $J \approx$ 6.8, 4 CH_3). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ = 1/1, 330 K): δ (vs TMS) 4.78, d, H-C(1′), $J_{1,2}$ = 7.5 Hz; the conformation of the newly formed C1′-O bond is β). ^{31}P NMR (300 MHz, pyridine; ^{1}H broad band decoupling): δ (vs phosphoric acid) 13.9 (s). $C_{68}H_{129}N_2O_{19}P$ (1309.8) Calcd: C, 62.38; H, 9.93; N, 2.14. Found: C, 61.80; H, 9.57; N, 2.60.

Due to solubility problems and the amorphous structures of **4** and **5**, no better C, H, N analysis could be obtained. However, the spectroscopic data are fully consistent with the proposed structures.

To prepare the lysine salt, a suspension of **4** or **5** in pyrogen-free H_2O was treated with an aqueous solution of 2 mol equiv of lysine. This mixture was sonicated and lyophilized. This lysine salt was used for all biological assays.

Determination of Reaction Kinetics. Test buffer (200 μL, 100 mM Tris·HCl (pH 7), 4000 mM NaCl, 2 mM EDTA, 2 mM DTE, and 0.5% Triton X-100) was mixed with 200 μL of a 5 mM solution of **1** or **2** and **3** in the same buffer as described for the enzymatic preparation. A 400-μL portion of enzyme solution was added and the mixture was incubated at 30 °C. At time zero, and after 12, 24, 36, 48, and 60 min, 100 μL of the test solution was mixed with 900 μL of an absorption mixture (70 mL of 500 mM KH_2PO_4, 20 mL of CH_3OH, 4 g of RP18 silica gel), shaken for 5 min and centrifuged. The supernatants were discarded. A 900-μL portion of a washing solution (85 mL of 500 mM KH_2PO_4, 5 mL of CH_3OH) was added to the pellets, the mixture was shaken for 5 min and centrifuged, and the supernatants were discarded again. To elute unreacted **3** from the RP18 silica gel, 900 μL of a desorption solution (88 mL of CH_3OH, 2 mL of CH_3COOH) was added; the mixture was shaken for 5 min and centrifuged. The optical density of the supernatant was measured at 260 nm.

LAL Assay. A test kit (Kabi-Vitrum, Stockholm, Sweden) was used according to the manufacturer's instructions. The test samples or standards were dissolved and diluted to the desired concentrations in endotoxin-free water. A 50-μL sample of each concentration was added to wells of a microtiterplate (Costar) and the plate was kept at 37 °C. Limulus amoebocyte lysate was dissolved in endotoxin-free water, prewarmed to 37 °C, and maintained at this temperature for ca. 10 min. The solution (50 μL) was then pipetted into the standard and test sample solutions. After carefully mixing, the microtiterplate was incubated for 13 min at 37 °C. (Exact timing is necessary for this step, each well

has to be incubated for exactly the same time. The sequence of pipetting has to be the same for this and all the following steps.) The chromogenic substrate was dissolved in prewarmed substrate buffer (37 °C) and kept at 37 °C. A 100-μL portion of this solution was added to the wells, the solutions were carefully mixed, and the microtiterplate was incubated for 3 min at 37 °C. The reaction was stopped by addition of 100 μL of 25% aqueous CH_3COOH. The optical density at 405 nm was measured using an ELISA reader. Results represent the mean of three values.

ODC Assay. ODC activity was measured by following a published procedure,[23] with some modifications. RAW-264 cells (ATCC, Rockville, MD) were suspended in DMEM-H21 (with 15 mM HEPES and 1 mM sodium pyruvate, no serum) to a final cell number of 5.5×10^5 cells/mL. For each sample, 10 mL of the cell suspension was transferred to 50 mL of cell culture flasks and incubated overnight at 37 °C/5% CO_2. Then 2000 units/mL murine γ-IFN (Sigma) and the test substances, diluted with pyrogen-free water to the desired concentration, were added. The flasks were incubated for 4 h at 37 °C with 5% CO_2. The supernatants were discarded. The cells attached to the surface of the flasks were washed off and suspended in 1 mL of the following buffer: 50 mM Tris·HCl, 0.01 mM EDTA, 2 mM DTE, 5 mM NaF, 0.1% Brij 35, 1 mM phenylmethanesulfonyl fluoride, and 60 μM pyridoxal phosphate. The cell suspensions were sonicated for 15 s each and centrifuged for 15 min in an Eppendorf centrifuge. The supernatants, containing the ODC, were frozen in liquid N_2. The frozen samples were thawed, and 190 μL of each sample was mixed with 5 μL of aqueous 0.2 mCi/mL [^{3}H]-L-ornithine hydrochloride (NEN, Boston, MA) and 7 μL of 1 mM L-ornithine hydrochloride. The solution was incubated at 37 °C for 60 min. A 150-μL portion of each sample was pipetted onto a 2.5 × 2.5 cm Whatman P-81 phosphocellulose filter paper. The filters were washed three times in 500 mL of 0.1 N NH_3 to remove unreacted [^{3}H]L-ornithine and dried under an IR lamp. The [^{3}H]putrescine content was determined by the addition of 10 mL of scintillation fluid (Lumagel, Baker) and subsequent reading in a β-counter. The stimulation values are expressed as percentage of ODC stimulation obtained with 5 ng/mL LPS. At this concentration of LPS maximal stimulation of ODC is observed.

Pyrogenicity Test in Rabbits. This test was carried out as described in the European Pharmacopeia II-1971.

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Synthesis and Antiallergic Activity of 11-(Aminoalkylidene)-6,11-dihydrodibenz[*b*,*e*]oxepin Derivatives

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A new series of 11-substituted 6,11-dihydrodibenz[*b*,*e*]oxepin-2-carboxylic acid derivatives was synthesized and demonstrated to be orally active antiallergic agents. These compounds are structurally related to **1** (KW-4994), which we had reported previously to be a new antiallergic agent. Most compounds synthesized exhibited potent inhibitory effects on 48-h homologous passive cutaneous anaphylaxis (PCA) in rats and on IgG_1-mediated bronchoconstriction in guinea pigs. Additionally, compounds possessing a terminal carboxyl group at the 2-position of the dibenz[*b*,*e*]oxepin ring system exhibited inhibitory effects on specific [^{3}H]pyrilamine binding to guinea pig cerebellum histamine H_1 receptors, whereas these demonstrated negligible effects on specific [^{3}H]QNB binding to rat striatum muscarinic acetylcholine M_1 receptors. Structure–activity relationship studies revealed that the following key elements were required for enhanced antiallergic activities: (1) a 3-(dimethylamino)propylidene group as the side chain at the 11-position, (2) a terminal carboxyl moiety at the 2-position, and (3) a dibenzoxepin ring system. Among the compounds synthesized, (*Z*)-11-[3-(dimethylamino)propylidene]-6,11-dihydrodibenz[*b*,*e*]oxepin-2-acetic acid hydrochloride (**16**) was selected for further evaluation. It had an ED_{50} value of 0.049 mg/kg po in the PCA test in rats and an ID_{50} value of 0.030 mg/kg po in inhibiting anaphylactic bronchoconstriction in guinea pigs. Furthermore, it had a K_i value of 16 ± 0.35 nM for the histamine H_1 receptor, while it exhibited negligible CNS side effects up to a dose of 600 mg/kg po. Compound **16** is now under clinical evaluation as KW-4679.

Introduction

Effective and orally active antiallergic agents with fewer side effects have been an attractive target for drug research in recent years.[2] Our research has been focused on the synthesis of a new series of benzoxepin derivatives and evaluation of their pharmacological properties.[3] In the course of our studies, we found that compound **1** (KW-4994, see Chart I) showed highly potent antiallergic activity with negligible central nervous system (CNS) side effects.[4]

Known antiallergic agents might be classified into two groups according to their chemical structures.[5] The basic agents such as **2** (ketotifen) elicit their antiallergic effects by mainly antagonizing histamine H_1 receptors. Acidic agents such as disodium cromoglycate (DSCG) predominantly work by inhibiting release of chemical mediators.

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