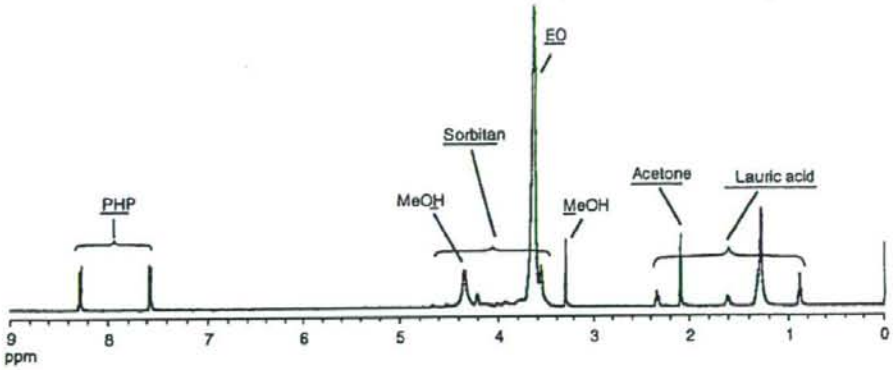

Figure 4. ^{1}H-NMR spectrum of polysorbate 20. The spectrum was obtained using the ECA500 system (500 MHz; JEOL). PHP was added as an internal standard. Signals of the four protons on the benzene ring of PHP were observed at δ_H values of 7.46–7.66 ppm and 8.18–8.38 ppm. Most of the EO signals of polysorbate 20 were observed in a large envelope between δ_H 3.40 and 3.85 ppm.

determination of the EO contents. Thus, in the current research, we used the EO signals between δ_H 3.40 and 3.85 ppm to determine the EO contents of polysorbates by NMR.

Determination of EO contents in polysorbates 20, 60, 65, and 80

Several reports have described the applications of qNMR to determine specific types of chemical compound, such as natural products, impurities, and polymers (Stefanova et al. 1988; Paula 2001; Jake et al. 2002; Wells et al. 2002; Paula et al. 2005). Recently, a practical set of parameters for qNMR has been discussed (Saito et al. 2004). Furthermore, qNMR using an internal standard has been suggested as a new way of determining the contents of surfactants with a relatively high throughput (Koike et al. 2004a, 2004b, 2005). To minimize quantitative errors, we used the qNMR conditions described by Koike and colleagues, as listed in Table I. In particular, the flip angle was set to 45°, and the spectral width was set at a value sufficient for the peak of interest to fall within 80% of its centre, because the signal intensities decreased towards both edges of the spectral window. The number of data points was set at 64 000 to enhance the resolution. The pulse delay was set at up to 30 s, as high-precision NMR can only be achieved when the pulse delay time is greater than the quintuple spin-lattice relaxation time ($>5 * T_1$). As qNMR is based on the fact that the signal intensities of a given resonance are directly proportional to the molar quantity of the nucleus within the sample, the EO signal intensity of polysorbates and four protons on the benzene ring of PHP were used to determine the EO contents. The total time taken to obtain one FID using these parameters was <10 min.

The weight percentage of the EO groups was calculated according to Equation 1.

$$\mathrm{EO(w/w\%)} = \frac{(I_{EO}/H_{EO} \times M_{EO}/W_{sample})}{(I_{standard}/H_{standard} \times M_{standard}/W_{standard})} \times 100. \quad (1)$$

Here, I_{EO} is the signal intensity of the EO group; H_{EO} is the number of protons of the EO group (four); M_{EO} is the partial molecular weight of the EO group (44); W_{sample} is the weight (mg) of the sample in 3 ml of NMR solvent including PHP as an internal standard; $I_{standard}$ is the total signal intensity of PHP; $H_{standard}$ is the number of protons on the benzene ring of PHP (four); $M_{standard}$ is the molecular weight of PHP (204); and $W_{standard}$ is the weight (mg) of PHP in 3 ml of NMR solvent.

We initially confirmed that the qNMR showed linearity between the intensity of the EO signal and the amount of polysorbate 20. Various amounts of the reagent-grade polysorbate 20 sample were analysed by ^{1}H-NMR under the conditions described in the Materials and methods and Table I. The NMR spectrum of polysorbate 20 with the internal standard is shown in Figure 4. The four protons of the PHP benzene ring were observed as two double–doublet signals at δ_H values of 7.46–7.66 ppm and 8.18–8.38 ppm, respectively. The ratio of the EO signal intensity was calculated as follows: intensity of EO/total intensities of four protons on PHP benzene ring. The relationship between EO/PHP and the amount of polysorbate 20 was linear ($R^2 = 0.9996$) in the range of 12.5–100 mg of polysorbate 20 in 3 ml of NMR solvent. Based on these results, we concluded

Table II. Determination of EO contents in polysorbates by qNMR.[a]

Sample name	MERCURY (400 MHz, VARIAN)			ECA500 (500 MHz, JEOL)		
	Entry	EO (%)	SD	Entry	EO (%)	SD
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	1	73.0		1	72.2	
	2	71.8		2	71.8	
	3	73.2		3	72.3	
	4	71.7		4	72.5	
	5	71.9		5	71.6	
				6	72.9	
				7	72.0	
				8	72.7	
				9	73.7	
	AV	72.3	0.7	AV	72.4	0.6
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	1	67.7		1	67.4	
	2	65.3		2	67.7	
	3	68.9		3	67.5	
	4	67.8		4	67.9	
	5	66.9		5	68.6	
	AV	67.3	1.3	AV	67.8	0.5
Polysorbate 65 (polyoxyethylene(20) sorbitan tristearate)	1	49.1		1	49.8	
	2	49.8				
	3	49.5				
	4	49.8				
	5	48.7				
	AV	49.4	0.5			
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	1	65.0		1	67.0	
	2	65.5				
	3	66.2				
	4	64.8				
	5	65.1				
	AV	65.3	0.6			

[a]Reagent-grade polysorbates were purchased from Wako Pure Chemical Industries, Ltd. "Entry" means that the same sample was measured repeatedly on different days.

that qNMR could quantitatively determine the EO contents of polysorbates.

In order to verify whether qNMR could accurately determine the EO contents of polysorbates, two different NMR instruments (MERCURY and ECA500, with magnetic field strengths of 400 and 500 MHz, respectively) were used to repeatedly measure the EO contents of reagent grade polysorbates 20, 60, 65, and 80, which are generally used as standards. The results are shown in Table II. Reproducible results were obtained from each sample using the MERCURY system. Furthermore, the results obtained by the two NMR instruments did not differ significantly (standard deviations = 0.5–1.3%). These findings confirmed that it was possible to determine the EO contents of polysorbates using this approach regardless of the NMR instrument employed.

Finally, to confirm the validity of qNMR, we determined the EO contents of the commercially synthesized polysorbates 20, 60, 65, and 80, which met the specifications of the JECFA. All of the EO contents of the polysorbates were within the limits described in the *Compendium of Food Additive and Flavoring Agent Specifications* (JECFA [internet]) (Table III). The qNMR method for determining the EO contents of polysorbates demonstrated in this paper thus represents a simple and rapid alternative to the classic titration method recommended by the JECFA, which does not require specific chemical reactions or sophisticated apparatus. Moreover, the qNMR method made it possible to distinguish between Polysorbates 60 and 80, which have the same stipulated value, by comparison with the ^{1}H-NMR spectra, as polysorbate 80 consisting of an unsaturated fatty acid only showed the signals of olefinic protons at δ_H 5.3 ppm. It is theoretically possible to determine the ratio of a substituted group in any molecule, or the quality of any compound, using the proposed qNMR method with an internal standard, provided that the target proton signals can be separated from those

Table III. EO contents in commercial polysorbates determined using qNMR.*

Name	Stipulated value	Brand	EO (%)	SD
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	70.0–74.0%	A	71.2	
		B	73.0	
		C	70.3	
		D	71.0	
		E	71.5	
		AV	71.4	1.0
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	65.0–69.5%	A	66.9	
		B	65.4	
		C	68.0	
		D	68.1	
		E	67.2	
		AV	67.1	1.1
Polysorbate 65 (polyoxyethylene (20) sorbitan tristearate)	46.0–50.0%	A	48.3	
		B	46.0	
		C	–	
		D	47.2	
		E	48.1	
		AV	47.4	1.1
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	65.0–69.5%	A	67.4	
		B	65.1	
		C	69.3	
		D	66.7	
		E	68.0	
		AV	67.1	1.6

*Brands A–E were purchased from five manufacturers. Brand C does not supply polysorbate 65.

of non-target groups and impurities. We are currently investigating the potential for this technique to determine various other compounds and polymers.

Conclusions

This research demonstrated that the EO contents of commercial polysorbates 20, 60, 65, and 80 could be readily determined using qNMR with an internal standard. Clear NMR data for the polysorbates were obtained from simple sample preparations. Two different NMR instruments validated the proposed method, and no significant differences were observed among the results. Moreover, the data obtained for commercial polysorbates 20, 60, 65, and 80 were in good agreement with the JECFA guidelines.

It is generally difficult to determine the amounts of substituted groups within polymers owing to their great diversity in molecular weights and structures. Classical methods require time-consuming preparation to set up the apparatus, and technically skilled operators. Furthermore, as there are no alternative methods to validate the results, they have to be accepted without verification. Our proposed qNMR is a rapid and simple analysis that provides the structural information of target compounds together. These advantages will reduce dramatically the time and manpower cost required, even if the NMR spectrometer and the solvents are expensive. qNMR is thus a valuable additional and/or alternative method, with a broad range of applications in quantitative analysis.

Acknowledgements

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RESEARCH ARTICLE

Highly-purified *Helicobacter pylori* LPS preparations induce weak inflammatory reactions and utilize Toll-like receptor 2 complex but not Toll-like receptor 4 complex

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Abstract

Helicobacter pylori is recognized as an etiologic agent of gastroduodenal diseases. Among toxic substances produced by *H. pylori*, LPS exhibits extremely low endotoxic activity as compared to the typical LPSs, such as that produced by *Escherichia coli*. We found that the LPS-low-responder stomach cancer cell line MKN28, which expresses Toll-like receptor 4 (TLR4) at extremely low levels, showed similar levels of interleukin-8 (IL-8) induction by *H. pylori* or *E. coli* LPS preparations. Weak IL-8 induction by *H. pylori* LPS preparations was suppressed by expression of a dominant negative mutant of TLR2 but not of TLR4. Data from luciferase reporter analysis indicated that cotransfection of TLR2–TLR1 or TLR2–TLR6 was required for the activation induced by *H. pylori* LPS preparations. In conclusion, the *H. pylori* LPS preparations significantly induce an inflammatory reaction via the receptor complex containing TLR2–TLR1 or TLR2–TLR6 but not that containing TLR4. The TLR2–TLR1 complex was preferentially recognized by the *H. pylori* LPS preparations over the TLR2–TLR6 complex. Whereas the magnitude of response to *H. pylori* LPS preparation was markedly less than that to *E. coli* LPS preparation in LPS-high-responder cells strongly expressing TLR4, it was comparable to that of *E. coli* LPS in low-responder cells expressing negligible amount of TLR4.

Introduction

Helicobacter pylori is a micro-aerophilic and spiral Gram-negative bacterium that can colonize gastric epithelial cells or gastric mucin. *Helicobacter pylori* is now recognized as a causative factor of chronic gastritis, gastroduodenal ulcer, gastric cancer and mucosa-associated lymphatic tissue lymphoma (Cover & Blaser, 1996; Parsonnet, 1998). Cell injury and inflammation caused by long-term chronic *H. pylori* infection is believed to underlie these diseases. Several bacterial products probably mediate cytotoxicity and the inflammatory reaction. Among these are vacuolating toxin (VacA), ammonium ions produced by urease, cytotoxin-associated gene A (CagA) protein, and the type IV secretion system, reviewed in Covacci *et al.* (1999) and Kusters *et al.* (2006). In general, Gram-negative bacterial LPSs are key inflammation inducers. However, *H. pylori* LPSs show extremely low endotoxic activity in comparison to typical Gram-negative bacterial LPSs, such as those from *Escherichia coli* (Nielsen *et al.*, 1994; Perez-Perez *et al.*, 1995; Yokota *et al.*, 2003; Hynes *et al.*, 2004).

Various substances produced by microorganisms are recognized by host receptors and trigger an innate immune response. Toll-like receptors (TLRs) have been shown to play important roles in the pattern recognition of microbial components, so the microbial substances recognized by TLRs are called 'pathogen-associated molecular pattern' (PAMP). At least 13 TLR family members in vertebrates and 10 TLRs in humans have been reported (Akira *et al.*, 2001; Barton & Medzhitov, 2002). These proteins recognize diverse PAMPs. Typical LPSs are recognized by a TLR4 complex consisting of TLR4, CD14 and MD2 expressed on host cells. Currently, it is controversial which TLR contributes to signal transduction by *H. pylori* LPS. Some reports

suggest that *H. pylori* LPS transduces signals via a TLR4 system. Kawahara *et al.* (2001) reported that TLR4 is required for induction of mitogen oxidase 1 by *H. pylori* LPS. Ishihara *et al.* (2004) reported that *H. pylori* infection upregulated MD2 expression, and upregulated MD2 increased cell surface expression of the MD2–TLR4 complex and the subsequent response to *H. pylori* LPS. Ogawa *et al.* (1997) reported that chemically synthesized *H. pylori* lipid A activates cytokine production in macrophages, and that the induction is inhibited by anti-TLR4 antibody. In contrast, Smith *et al.* (2003) and Lepper *et al.* (2005) reported that TLR2 is required for signal transduction induced by *H. pylori* LPS. Notably, Lepper *et al.* (2005) suggested that LPSs derived from some *H. pylori* strains antagonize TLR4 signaling activated by a typical LPS. Here, we investigated which TLR responded to *H. pylori* LPS in stomach adenocarcinoma cell lines, whose response to LPS is very weak and comparable in the case of both *E. coli* and *H. pylori* LPS.

Materials and methods

Bacterial strains and preparation of LPS

Helicobacter pylori clinical strains were isolated from the biopsy specimens obtained from patients with chronic gastritis (CG), gastric ulcer (GU), duodenal ulcer (DU), and gastric cancer (CA). After fewer than three passages of laboratory culture, these bacteria were grown on brucella broth (BBL, Cockysville, MD) supplemented with 10% (v/v) horse serum at 37 °C for 2 days in micro-aerophilic conditions, using the Campypack system (BBL). The organisms were collected by centrifugation (10 000 *g*, 30 min), washed with de-ionized water twice, and lyophilized. The conventional LPS preparation was obtained from the lyophilized cells by the hot phenol/water method (Yokota *et al.*, 2000). In some experiments, a highly purified LPS preparation, which was further purified in several steps as follows, was used, due to the potential influence of contaminants. The conventional LPS preparation was treated with 10 $\mu g\,mL^{-1}$ DNase I (Roche Diagnostic, Tokyo, Japan), 10 $\mu g\,mL^{-1}$ RNase A (Qiagen, Hilden, Germany), 2 $\mu g\,mL^{-1}$ lipoprotein lipase from bovine milk (Sigma-Aldrich, St Louis, MO) and 10 $\mu g\,mL^{-1}$ lipoprotein lipase from *Pseudomonas* sp. (Sigma-Aldrich) at 37 °C for 12 h in 50 mM sodium phosphate buffer (pH 7.2). Finally, proteinase K (Wako Junyaku, Tokyo, Japan) was added to the reaction mixture at a concentration of 20 $\mu g\,mL^{-1}$, and the mixture was incubated at 60 °C for 4 h. The resulting material was subjected to Octyl-Sepharose column chromatography (GE Healthcare Bio-Science, Piscataway, NJ). The column was equilibrated with 0.5 M NaCl in 0.1 M sodium/acetate buffer (pH 4.7). The column was washed with 30% propanol, and then LPS was eluted with a linear gradient of 30–60% propanol. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining as previously described (Hitchcock & Brown, 1983). For buffer exchange, the pooled LPS fraction was applied to a PD-10 desalting column (GE Healthcare Bio-Science) equilibrated with 0.2% triethylamine. The material eluted at void volume was pooled, lyophilized and used as a highly purified LPS preparation.

Campylobacter jejuni strains 81–176 and OH4832, which were isolated from a gastroenteritis patient and a Guillain–Barré syndrome patient, respectively, were provided by Dr Misawa (Miyazaki University, Miyazaki, Japan). *Campylobacter jejuni* LPS was prepared by phenol/water extraction (Yokota *et al.*, 2000). LPSs derived from *E. coli* O111:B4 (wild type) and D31m4 (Re mutant) were purchased from LIST Biochemical Laboratories (Campbell, CA).

Cell lines

The human gastric carcinoma cell lines MKN28 and MKN45 were obtained from the Japanese Collection of Research Biosources (JCRB, Ibaraki, Japan). The human uroepithelial cell line T24 and the human monocytic cell line THP-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human embryonic kidney 293 cell line was obtained from the Human Science Research Resource Bank (Tokyo, Japan). MKN28, MKN45, T24 and 293 were routinely cultured in Dulbecco's modified minimum essential medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U mL^{-1}), and streptomycin (100 $\mu g\,mL^{-1}$). THP-1 was cultured in RPMI-1640 containing 10% (v/v) FBS, penicillin (100 U mL^{-1}), and streptomycin (100 $\mu g\,mL^{-1}$).

Expression plasmids

Human cDNAs encoding TLR2 and MD2 were prepared from RNA derived from THP-1 cells by reverse transcription (RT)-PCR. Human TLR4 cDNA was prepared from human spleen total RNA (OriGene Technologies, Rockville, MD) by RT-PCR. Human CD14 cDNA was kindly provided by Dr Shunsuke Yamamoto (Medical College of Oita, Oita, Japan). cDNAs encoding human TLR2, TLR4, MD2 and CD14 minus signal peptide sequences were subcloned into the downstream portion of a modified pcDNA3 vector (Invitrogen, Carlsbad, CA). The product generated from this vector contains the coding sequence for the preprotrypsin signal peptide sequence preceding the N-terminal equine infectious anemia virus tag epitope. Expression plasmids encoding TLR5, dominant negative TLR2 and dominant negative TLR4 were kindly donated by Dr M.F. Smith (University of Virginia) (Smith *et al.*, 2003). Dominant negative mutants had deletions of the C-terminal portion of the molecules

(TLR2 truncated at amino acid 670, and TLR4 at amino acid 700). Expression plasmids encoding TLR1 and TLR6 were kindly donated by Dr Ken-ichiro Shibata (Hokkaido University, Sapporo, Japan) (Fujita *et al.*, 2003).

Enzyme-linked immunosorbent assay (ELISA)

The amounts of interleukin-8 (IL-8) in culture supernatants were determined with an ELISA Development kit for human IL-8 (R&D Systems, Minneapolis, MN).

Western blotting analysis

SDS-PAGE and Western blotting were carried out as described previously (Yokota *et al.*, 2000). A mouse anti-CD14 monoclonal antibody (UCH-M1) and a rabbit anti-TLR2 polyclonal antibody (H-175) were purchased from Santa Cruz Bioechnology (Santa Cruz, CA). Rabbit anti-TLR1 polyclonal antibodies and mouse anti-TLR6 monoclonal antibodies were purchased from Abcam (Cambridge, UK). Alkaline phosphatase-labeled goat anti-mouse or anti-rabbit IgG antibodies were purchased from BioSourse International (Camarillo, CA), and used as secondary antibodies. Specific binding was detected using tetrazolium bromochloroindolylphosphate/Nitro Blue Tetrazolium as a developing substrate.

Real-time RT-PCR

Total RNA was isolated from cells using an RNeasy Mini kit (Qiagen). The cDNA was prepared from total RNA with a Reverse Transcription System kit (Promega, Madison, WI) using random primers (GE Healthcare Bio-Science). Real-time PCR was performed using a TaqMan Gene Expression Assay kit (Applied Biosystems, Foster City, CA) with a 7300 Fast Real-Time PCR system (Applied Biosystems). mRNA levels of TLR2, TLR4, CD14, MD2 and glyceraldehyde-3-phosphate dehydrogenase were determined. The mRNA levels were normalized with the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase.

Flow cytometry

Flow cytometry was performed using a FACSCalibur (Beckton-Dickinson, San Jose, CA). Phycoerythrin-labeled mouse monoclonal antibodies against TLR2 (clone TL2.1), TLR4 (HTA125) and CD14 (61D3), and their isotype controls, phycoerythrin-labeled mouse monoclonal IgG_1 and IgG_{2a}, were purchased from eBioscience (San Diego, CA).

Luciferase reporter gene assay

A luciferase reporter plasmid carrying the promoter region of E-selectin (pELAM-L) was constructed by inserting a PCR fragment (−730 to +52) of the E-selectin promoter into the SacI–HindIII site of the pGL3 Basic vector (Promega, Madison, WI). After plating of 293 cells in 12-well plates (2×10^5 well^{-1}), cells were transfected by the calcium phosphate precipitation method with the indicated expression plasmids (0.02 μg of TLR1, TLR2, and TLR6), together with 0.02 μg of CD14 expression plasmid, 0.1 μg of pELAM-L luciferase reporter plasmid, and 0.0025 μg of phRL-TK (Promega) for normalization. In some experiments, CD14 expression vector was eliminated, as described in 'Results'. After 24 h, cells were stimulated with LPS for 6 h. Reporter activity was measured according to the manufacturer's (Promega) recommendations.

Pam_3CSK_4 (as a TLR2–TLR1 agonist), MALP-2 (as a TLR2–TLR6 agonist) and recombinant flagellin protein of *Salmonella muenchen* (as a TLR5 agonist) were purchased from Bachem (Bubendorf, Switzerland), Alexis (Lausen, Switzerland), and Calbiochem (San Diego, CA), respectively.

Results

Relationship of TLR4 expression levels to LPS responsiveness in various cell lines

We examined responsiveness to *H. pylori* LPS preparations as compared with *E. coli* LPS preparations of stomach adenocarcinoma cell lines (MKN28 and MKN45) and a uroepithelial cell line (T24). The *H. pylori* LPSs used in this study were from six strains. LPS preparations derived from strains GU2 and DU2 are smooth types carrying the highly antigenic epitope. LPS preparations derived from strains CA2 and CA6 are smooth types carrying the weakly antigenic epitope, and are frequently found in strains derived from gastric cancer patients. LPS preparations derived from CG10 and DU8 are rough types lacking the O-polysaccharide chain (Yokota *et al.*, 1997; Yokota *et al.*, 2000).

The LPS-high-responder cell line T24 produced high levels of IL-8 after stimulation with an *E. coli* wild-type LPS preparation (Fig. 1a). The *E. coli* rough LPS preparation was less active than the smooth LPS preparation. *Helicobacter pylori* LPS preparations from all strains showed very weak (500-fold less than *E. coli* smooth LPS) IL-8 induction. The weak response to the *H. pylori* LPS preparation was consistent with previous reports (Nielsen *et al.*, 1994; Perez-Perez *et al.*, 1995; Yokota *et al.*, 2003; Hynes *et al.*, 2004). MKN28 showed a weak response to the *E. coli* LPS preparation (Fig. 1c). Interestingly, similar levels of IL-8 induction were observed with *E. coli* and *H. pylori* LPS preparations. MKN45 cells showed responses between those of T24 and MKN28 cells (Fig. 1b). Here, we also examined *H. pylori* LPS from which probable contaminants were extensively removed. Such contaminants were destroyed by treatment with proteinase K, DNase, RNase and lipoprotein lipases,

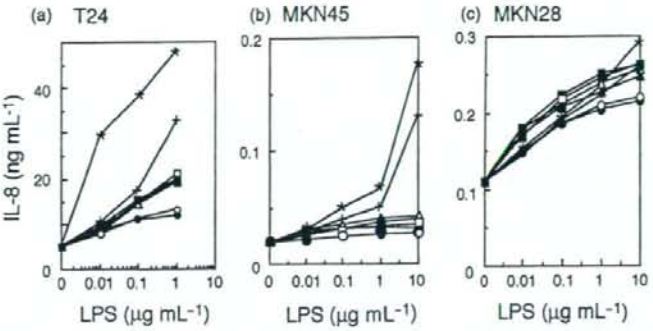

Fig. 1. IL-8 induction by LPS derived from *Helicobacter pylori* and *Escherichia coli* in the human uroepithelial cell line T24 and in human gastric carcinoma lines MKN28 and MKN45. Cells were treated with various concentrations of LPS for 24 h. Concentrations of IL-8 in the resulting culture supernatant were determined by ELISA. The LPSs used were derived from *Helicobacter pylori* strain GU2 (○), DU2 (●), CA2 (△), CA6 (▲), CG10 (□), and DU8 (■), and *Escherichia coli* O111 : B4 wild-type (*) and Re mutant (+) strains. The data represent results for the conventional LPS preparations. Similar results were obtained when the highly purified LPS preparation was used.

and the LPSs were further subjected to hydrophobic interaction chromatography. The lipoprotein lipase derived from *Pseudomonas* treatment decreased IL-8 induction by MALP-2 and Pam_3CSK_4 by $< 5\%$ in THP-1 cells. Lipoprotein lipase derived from bovine milk treatment decreased IL-8 induction by Pam_3CSK_4 by $< 2\%$ (data not shown). Hashimoto *et al.* (2004) showed that lipoprotein lipase could reduce the activity of contaminated lipoprotein in a preparation of *Pseudomonas gingivalis* LPS isolated with a conventional method. We further performed hydrophobic interaction chromatography on an Octyl-Sepharose column. This chromatographic method has been shown to be suitable for removal of contaminants in the conventional LPS preparation (Hashimoto *et al.*, 2004; Lepper *et al.*, 2005). After enzyme treatment and hydrophobic interaction chromatography, our LPS preparation did not show significantly altered reactivity (data not shown). This suggests that the activity of our *H. pylori* LPS preparation obtained by the conventional method is due to LPS itself and not to contaminants. *Helicobacter pylori* LPS preparations from GU2 and DU2 showed weaker IL-8 induction than did the *H. pylori* LPS preparation from other strains in all cells tested (Fig. 1).

We analyzed the expression of LPS receptor components and other TLRs in these cell lines. Cell surface expression of TLR4, CD14 and MD2 is required for recognition and signal transduction of a typical LPS, such as *E. coli* LPS, by host cells. TLR2 and MD2 mRNA levels were comparable in all three cell lines. TLR4 and CD14 mRNA levels were in the following order, from higher to lower: T24 > MKN45 > MKN28 (Fig. 2a). Cell surface protein expression levels as determined by flow cytometry were consistent with mRNA levels (Fig. 2b). This expression level of TLR4 and CD14 was positively correlated with responsiveness to the *E. coli* LPS preparation. TLR1 and TLR6 mRNA levels were in the following order, from higher to lower: MKN45 > T24 > MKN28. In particular, TLR6 mRNA was not detectable in MKN28 cells.

Effect of dominant negative mutant of TLR4 on IL-8 production induced by *E. coli* and *H. pylori* LPS preparations

To examine the contribution of TLR4 to *E. coli* or *H. pylori* LPS-induced IL-8 production, we overexpressed dominant negative mutants of TLR4 or TLR2 in LPS-high-responder T24 cells (Fig. 3). *Escherichia coli* LPS-induced IL-8 production was markedly suppressed by expression of dominant negative TLR4 but not by dominant negative TLR2. On the other hand, IL-8 production induced by the *H. pylori* LPS preparation was significantly suppressed by dominant negative TLR2 but not by dominant negative TLR4. These results indicate that TLR2, but not TLR4, is necessary for IL-8 induction by the highly purified *H. pylori* LPS preparations.

Effect of *E. coli*, *C. jejuni* and *H. pylori* LPS preparations on nuclear factor-κB (NF-κB) activation in 293 cells expressing TLRs and related molecules

Next, we examined NF-κB activation induced by *H. pylori*, *C. jejuni* and *E. coli* LPS preparations in 293 cells coexpressing TLR4, CD14 and MD2 as determined by a luciferase reporter assay (Fig. 4). LPS preparations derived from *C. jejuni*, which, like *Helicobacter*, belongs to the *Campylobacterales* family, activated NF-κB to a similar extent as *E. coli* LPS preparations. *Helicobacter pylori* LPS preparations had little effect on NF-κB activation. The results indicate that the *H. pylori* LPS preparation does not transduce cellular signaling via TLR4, in contrast to other Gram-negative bacterial LPSs.

To address the contribution of other TLRs to *H. pylori* LPS signal transduction, we expressed various TLRs and related molecules and undertook similar assays in 293 cells, which lack TLRs (Fig. 5). 293 cells expressing TLR2, TLR4–MD2 or TLR5, plus CD14 did not respond to *H. pylori* LPS preparations, even at a concentration of $10\,\mu g\,mL^{-1}$. Next, we coexpressed TLR1 or TLR6 with

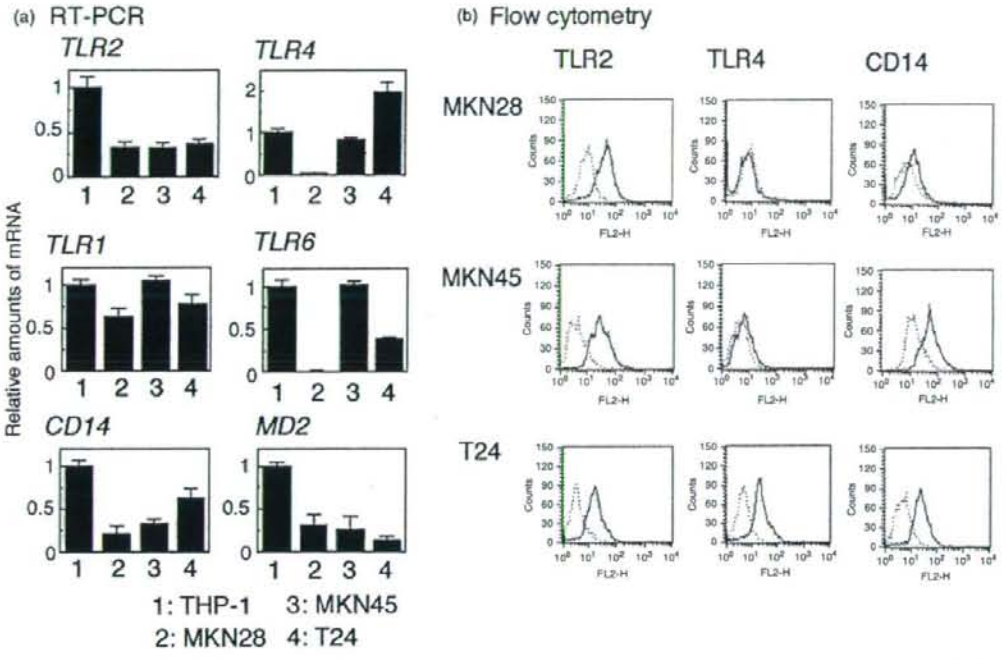

Fig. 2. Expression of (a) mRNA levels (by real-time RT-PCR) of TLR2, TLR4, TLR1, TLR6, CD14 and MD2, and (b) cell surface protein levels (by flow cytometry) of TLR2, TLR4 and CD14 in MKN28, MKN45 and T24 cells. THP-1 cells were used as positive controls. The relative amounts of mRNA levels in relation to THP-1 cells (expression levels in THP-1 cells taken as 1) were calculated. (b) Hatched lines indicate cells incubated with isotype-matched control antibodies.

TLR2 plus CD14. CD14 expression is required for recognition of ligands for TLR2–TLR1 and TLR2–TLR6 (Schroder *et al.*, 2004). We also found that coexpression of CD14 significantly elevated TLR2–TLR1- and TLR2–TLR6-mediated responses (data not shown). We examined the expression levels of TLR2, TLR1, TLR6 and CD14 in each transfectant by Western blotting (Fig. 5b). The expression levels of each of these molecules was not affected by the coexpression of other TLRs and CD14. This suggested that differences in responsiveness of these TLR systems were not due to different expression levels of TLRs and CD14. Significant NF-κB activation in response to *H. pylori* LPS was observed in 293 cells expressing TLR2–TLR1 or TLR2–TLR6. The TLR2–TLR1 system showed stronger activation than the TLR2–TLR6 system. Both TLR2–TLR1 and TLR2–TLR6 agonists, as positive controls, responded well. The results suggested that the *H. pylori* LPS preparations preferentially transduced signals via TLR2–TLR1 rather than TLR2–TLR6. Significant activation via the TLR2–TLR1 system was observed for LPS preparations derived from CA2 and GU2 strains at concentrations of 1 and 10 $\mu g\,mL^{-1}$, respectively. The CA2 LPS preparation was a more potent NF-κB activator (Fig. 5) and IL-8 inducer (Fig. 1) than the GU2 LPS preparation. However, the magnitude of NF-κB activation by *H. pylori* LPS preparations was markedly less than that of a conventional agonist (Fig. 5). Thus, the degree of NF-κB activation by the *H. pylori* LPS preparation appeared to correlate with the degree of IL-8 induction. These results suggest that the *H. pylori* LPS preparation weakly activates NF-κB via hetero-oligomers containing TLR2 but not TLR4, and transduce a signal preferentially via TLR2–TLR1 rather than TLR2–TLR6.

Discussion

Helicobacter pylori LPS exhibits extremely weak endotoxic activities, such as NF-κB-mediated inflammatory reactions. LPS preparations from several bacteria, such as *Porphyromonas gingivalis* (Ogawa *et al.*, 1997), *Bacteroides fragilis* (Lindberg *et al.*, 1990), *Chlamydia trachomatis* (Heine *et al.*, 2003) and *Rhodobacter* species (Rose *et al.*, 1995; Weckesser & Mayer, 1988) also show weak endotoxic activity. Whereas the typical LPS transduces signals via the TLR4 complex containing MD2 and CD14, the identity of signal-

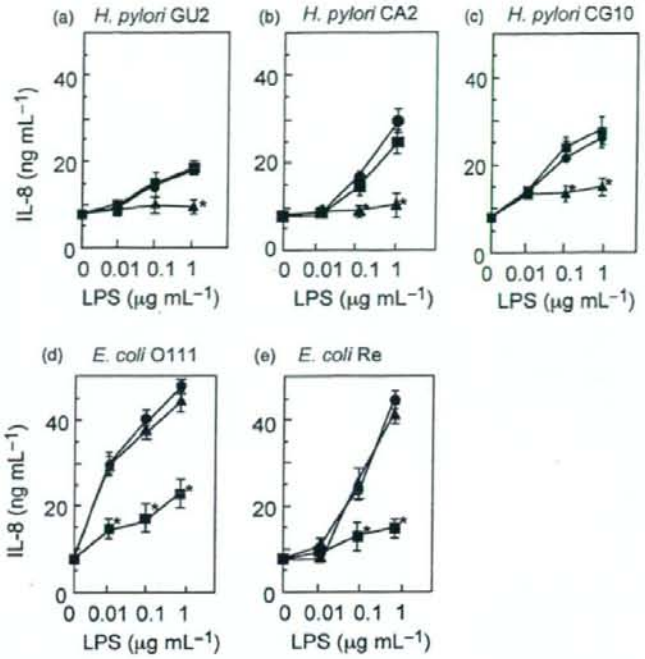

Fig. 3. Effect of overexpression of dominant negative mutants of TLR2 or TLR4 on LPS-induced IL-8 induction in T24 cells. Expression plasmids encoding dominant negative mutants of TLR2 (▲) or TLR4 (■), or empty plasmid (●), were transfected into T24 cells. After 24 h of transfection, cells were treated with *Helicobacter pylori* LPS derived from strain GU2 (a), strain CA2 (b) or strain CG10 (c), or LPS from *Escherichia coli* O111 : B4 (d) or *Escherichia coli* Re mutant (e). Concentrations of IL-8 in the resulting culture supernatants were determined by ELISA. Experiments were done in triplicate. The results were expressed as fold induction (mean values ± SD), relative to the value obtained from control experiments without LPS stimulation. $^*P < 0.01$ compared to LPS-untreated control. The data represent results for the highly purified LPS preparations. Similar results were obtained when the conventional LPS preparations were used.

transducing receptor(s) of weak endotoxic LPS is controversial. For example, Erridge *et al.* (2004) have reported that LPS with the low endotoxic activity shown above commonly transduces signals via TLR2. In contrast, Hashimoto *et al.* (2004) indicated that contaminating lipopeptide in a *P. gingivalis* LPS preparation is an agonist of TLR2, and LPS is a weak agonist of a TLR4-containing complex.

In the case of *H. pylori*, bacterial components other than LPS can induce NF-κB-mediated inflammatory reactions. As a TLR-independent pathway, the type IV secretion system, consisting of proteins encoded by genes located in

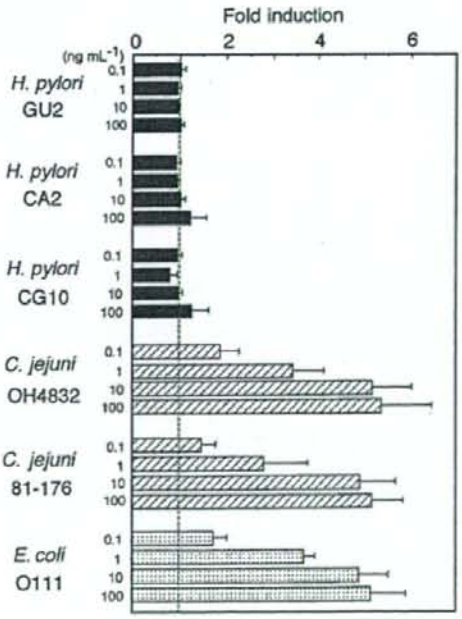

Fig. 4. Reporter gene assay of NF-κB activation by *Helicobacter pylori*, *Campylobacter jejuni* and *Escherichia coli* LPS using 293 cells expressing TLR4, CD14 and MD2. Expression plasmids of TLR4, CD14 and MD2 and reporter plasmids of pELAM-L and phRL-TK were cotransfected into 293 cells. After 24 h, cells were stimulated with the conventional LPS preparation derived from *Helicobacter pylori* (strains GU2, CA2, and CG10), *Campylobacter jejuni* (strains OH4832 and 81–176) or *Escherichia coli* O111 : B4 for 6 h. The reporter activity was measured. The experiments were done in triplicate. The results were expressed as fold induction (mean values ± SD), relative to the value obtained from control experiments without LPS stimulation.

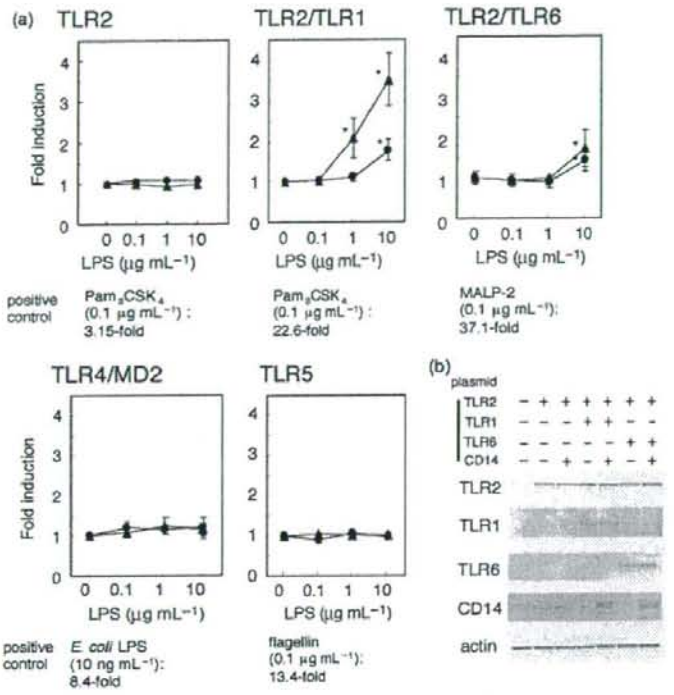

Fig. 5. Reporter gene assay of NF-κB activation by *Helicobacter pylori* LPS using 293 cells expressing various TLR systems. (a) Expression plasmids indicated at the top of each graph plus expression plasmid of CD14, and reported plasmids (pELAM-L and phRL-TK), were cotransfected into 293 cells. After 24 h, cells were stimulated with the conventional LPS preparation derived from *Helicobacter pylori* strain GU2 (●) or strain CA2 (▲) for 6 h. Positive control experiments were carried out with Pam_3CSK_4 (for TLR2 and TLR2–TLR1), MALP-2 (for TLR2–TLR6), LPS derived from *Escherichia coli* O111:B4 (for TLR4–MD2) or flagella derived from *Salmonella* (for TLR5) as a stimulant. The reporter activity was measured. The experiments were done in triplicate. The results were expressed as fold induction (mean values ± SD), relative to the value obtained from control experiments without LPS stimulation. $^*P < 0.01$ compared to LPS-untreated control. (b) Protein expression levels of TLR2, TLR1, TLR6 and CD14 in transfectants with plural expression plasmids. 293 cells were transfected with the expression plasmids indicated at the top of the figure. After 24 h of transfection, cells were lysed and assessed by Western blotting.

the *cag* pathogenicity island, including CagE, contributes to NF-κB activation (Glocker *et al.*, 1998; Naumann *et al.*, 1999). Viable bacterial cells are required for type IV secretion system-dependent NF-κB activation. Although flagella derived from various bacteria activate NF-κB via the TLR5-dependent pathway, *H. pylori* flagella cannot activate such systems (Andersen-Nissen *et al.*, 2005). Other protein components, such as heat shock protein 60 (Takenaka *et al.*, 2004) and urease B (Beswick *et al.*, 2006), act as NF-κB activators and are suggested to be TLR agonists.

Helicobacter pylori LPS is also believed to be a TLR agonist. It is still controversial which receptor is recognized by *H. pylori* LPS. Lepper *et al.* (2005) and Smith *et al.* (2003) reported that TLR2 is essential for signal transduction activated by *H. pylori* LPS. On the other hand, Kawahara *et al.* (2001), Ishihara *et al.* (2004) and Hashimoto *et al.* (2004) reported that TLR4 plays an essential role in LPS-induced inflammatory responses. Maeda *et al.* (2001) indicated that mechanisms of NF-κB activation differ among cell types. Monocyte–macrophage (THP-1) cells respond to *H. pylori* in a TLR4–CD14 system-dependent manner. In contrast, epithelial (MKN45) cells show TLR-independent and *H. pylori cag*-dependent responses to *H. pylori*. Maeda *et al.* concluded that monocyte–macrophage cells are activated by *H. pylori* via the TLR4 system, and this might be dependent on LPS. In contrast, epithelial cells are mainly activated by viable *H. pylori* cells, and this is dependent on the type IV secretion system but not on LPS.

There might be several reasons for such contradictions. The first might be contamination of LPS preparations by lipoproteins, proteins, nucleic acids and other bacterial LPSs. Lipoproteins and other bacterial LPSs may stimulate TLR2 and TLR4 signals, respectively. Furthermore, bacterial nucleic acids may stimulate other TLRs, such as TLR9 (Akira *et al.*, 2001; Barton & Medzhitov, 2002). The second reason might be differences in the experimental systems used, such as different cell lines and strategies used to identify each TLR. For example, the strategies are transfection of dominant negative mutants, suppression by neutralizing antibodies, and transfection of TLR-expression plasmids into TLR-deficient cell lines, such as 293 cells. The third, and possibly most important, reason is heterogeneity of the structures of *H. pylori* LPS molecules within a given preparation. This could result from strain differences and/or bacterial culture condition. The chemical structure of lipid A, e.g. the number and chain length of fatty acid residues and the presence of phosphate groups, derived from these bacteria differs from that of a typical enterobacterial LPS (Netea *et al.*,

2002). Tran *et al.* (2005) reported that the lipid A portion of *H. pylori* LPS undergoes several structural modifications through the action of specific modifying enzymes. The 'remodeling' of LPS should generate structural heterogeneity in lipid A among strains and culture conditions.

Here, we used highly purified preparations of *H. pylori* LPS, from which contaminants were removed by extensive purification. Such contaminants were removed by proteinase K, DNase, RNase and lipoprotein lipases, and the LPS was further subjected to hydrophobic interaction chromatography. After enzyme treatment and hydrophobic interaction chromatography, our LPS preparation did not show altered reactivity, indicating that the activity is due to LPS itself and not to contaminants. Our results clearly suggest that inflammatory signal transduction mediated by *H. pylori* LPS in our highly purified preparation requires TLR2 but not TLR4. Complexes containing TLR2 and TLR1 were more efficient signal-transducing receptors for *H. pylori* LPS than complexes containing TLR2 and TLR6. The similar activities were observed for six strains, which had various characters, such as smooth/rough phenotypes and antigenicity of the polysaccharide portion. In the present study, we cannot completely rule out the possibility that very small amounts of TLR agonists were present in the highly purified LPS preparations. Furthermore, we have not examined different LPS preparations from cells grown under various culture conditions, and determined the chemical structures of lipid A portions. However, the TLR2-dependent activity that we observed seemed to be common to LPS preparations from various strains of *H. pylori*. The structural requirements for the TLR2-dependent activity need to be clarified.

The magnitude of response to a typical LPS, such as that from *E. coli*, was correlated with expression levels of typical LPS receptor complexes, which contain TLR4, CD14 and MD2. Higher expression levels of TLR4 and CD14 were seen in LPS-high-responder cells, such as T24. On the other hand, TLR2 expression levels were similar among all cell lines used in this study. In conclusion, *H. pylori* LPS is a weak but significant inducer of inflammatory reactions. The magnitude of the inflammatory response stimulated by *H. pylori* LPS is comparable to that of typical LPS in LPS-low-responder cells (e.g. MKN28), which express TLR4 at very low levels.

Acknowledgements

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Note

Synthesis of Lipid Derivatives of Pyrrole Polyamide and Their Biological Activity

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Novel fatty acyl and phospholipid derivatives of pyrrole polyamide were synthesized. Their cytotoxicity against a cancer cell line of MT-4 cells and those infected by human immunodeficiency virus (HIV) was examined. Although no anti-HIV activity was found, their cytotoxicitty against the cancer cells was significantly enhanced by introducing a lipophilic group into the pyrrole polyamide.

Key words: pyrrole polyamide; lipid; phospholipid; cancer cell; human immunodeficiency virus (HIV)-II

Since the naturally occurring pyrrole polyamide, netropsin, as an antibiotic was reported by Kopka *et al.*,[1,2] a number of pyrrole polyamide analogues has been synthesized so far. Subsequently, their highly sequence-specific binding to DNA[3] has intensively triggered the design of novel functional pyrrole polyamides,[4] and the search for new biological functions including anti-cancer[5] and anti-HIV[6] activities.

As the major components of polyunsaturated fatty acids (PUFA) in marine fish oil, docosahexaenoic acid (DHA) and icosapentaenoic acid (EPA) have a non-conjugated all-*cis*-polyunsaturated olefinic structure. They are known to exhibit a variety of biochemical and physiological functions including enhanced cell membrane permeability,[7] growth regulation and apoptosis-inducing capability to cancer cells,[8–10] cytotoxicity enhancement for some anti-cancer drugs against cancer cells[11,12] and potential anti-malarial activity.[13] Regarding the effect of the lipid modification of bioactive compounds, Zerouga *et al.* have reported that methotrexate, a cytotoxic drug, conjugated to phosphatidylcholine (PC) having a docosahexaenoyl group showed higher anti-proliferative activity against murine leukemia cells than one having a stearoyl group.[14] In our previous study, conjugates of qunine with fatty acid were found to show higher cytotoxicity against tumor cell line FM3A[15] than qunine itself. Parang *et al.* have extensively reviewed the relationship between the lipid modifications of 3′-azido-2′,3′-dideoxythymidine (AZT) and anti-HIV activity.[16] In the present study, novel fatty acyl derivatives of pyrrole polyamide were synthesized by using stearic acid (**3**, a typical saturated fatty acid rich in mammal fats), linoleic acid (**4**, a typical n-6 type of dienoic acid rich in plant lipids) and icosapentaenoic acid (**5**, a typical n-3 type of pentaenoic acid rich in fish oil), and the cytotoxicity of the conjugates was examined by using MT-4 cells.

Pyrrole polyamide has so far been synthesized by a reaction sequence involving the nitration of pyrrole, reduction of the nitro group to an amino group and condensation of the amine with nitro-pyrrole carboxylic acid.[17] This route, however, involves some intermediates having a nitro group that is known to cause allergic symptoms. To minimize the number of these intermediates, a different approach was investigated to obtain a key intermediate (**2**). Briefly, the route involves trichloroacetylation at the 2-position of *N*-methylpyrrole, nitration at the 4-position of the pyrrole nucleus, conversion of the trichloroacetyl group to a methoxycarbonyl group, reduction of the nitro group to an amino group, condensation of the amine with *N*-methylpyrrole

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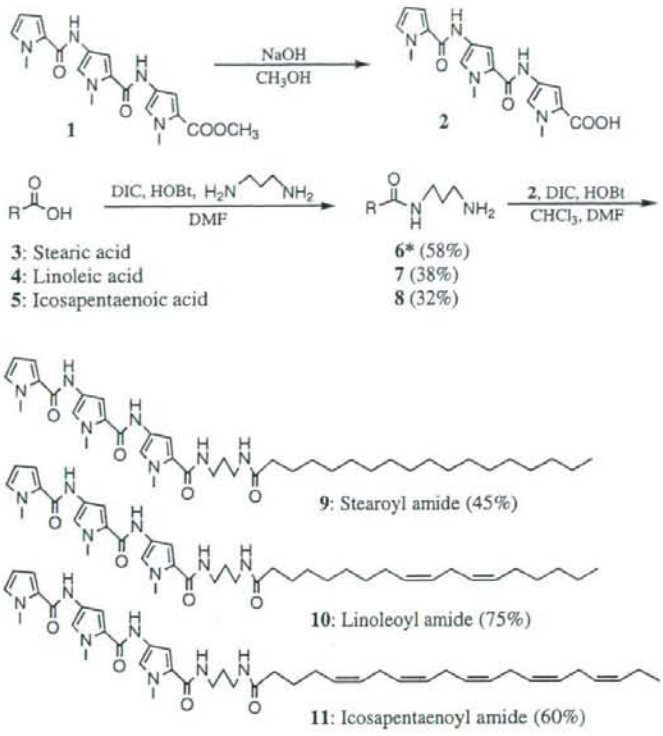


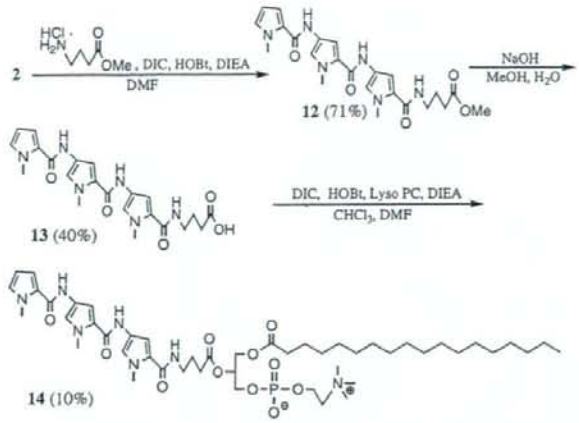
Scheme 1. Synthesis of Fatty Acyl Derivatives (**9**)–(**11**).
*Ref. 21. DIC, Diisopropylcarbodimide; HOBt, Hydroxybenzotriazole

2-carboxylic acid, hydrolysis of the methyl ester to a carboxylic acid, condensation of this acid with *N*-methyl-4-aminopyrrole 2-carboxylic acid methyl ester that had been prepared as already described to afford an intermediate **1**,[18] and finally hydrolysis of the methyl ester to afford **2**.

Acylated pyrrole polyamide (**9**–**11**) were synthesized by condensation of fatty acid half amides (**6**–**8**) with the carboxylic acid (**2**) respectively (Scheme 1) as described in the experimental section.

A phospholipid derivative (**14**) was synthesized by using the same intermediate **2** (Scheme 2). In this case, methyl γ-aminobutylate was introduced into **2** as a spacer giving ester **12**, which was hydrolyzed to **13**. Due to its instability, a product **13** was submitted as such to condensation with a lyso PC having a stearoyl group at *sn*-1 position. Silica gel chromatography afforded the desired compound **14** whose structural integrity was confirmed by ^{1}H-NMR and ESI MS data. For further structural confirmation, hog pancreatic phospholipase A2 was applied in an acetate buffer (pH 8.4) to substrate **14** at room temperature for 12 h. A TLC analysis showed that the lyso PC and pyrrole polyamide **13** having the spacer had been liberated by the enzymatic reaction. This finding might give an opportunity to use this enzyme as a molecular switch to liberate the pyrrole polyamide at the right time when it should play some roles in biological systems.[4]

As a preliminary test of biological activity in the present study, lipid derivatives **9**–**11** and **14** were examined for their *in vitro* cytotoxicity against cultured MT-4 cells. The result showed that the order of their concentration for complete growth inhibition of cultured MT-4 cells was stearoyl derivative **9** (45 μM) < icosapentaenoyl derivative **11** (176 μM) < linoleoyl derivative **10** (≥181 μM) < pyrrole amide methyl ester **1** (≥1304 μM) as a control. This experiment demonstrated for the first time that lipid modification of a pyrrole polyamide remarkedly enhanced its cytotoxicity, and derivative **9** with a saturated acyl group appeared to be more active than the unsaturated type. The same tendency was also observed for the concentration range of partially inhibitive and non-inhibitive cases. Although phospholipid derivative (**14**) enhanced the cytotoxicity (≥260 μM) to some extent, the activity was lower than those by acyl derivatives **9**–**11**. Combining all the results, lipid modification of the pyrrole polyamide was found to significantly enhance the cytotoxicity against cancer cell line MT-4 cells, and the effect appeared to be higher with the saturated acyl derivative than with the



Scheme 2. Synthesis of Phospholipid Derivatives (**14**).
DIEA, Diisopropylethylamine

unsaturated types. No anti-HIV effect was, however, appearent by the microplate method[19] and Magic 5 method[20] for any of the pyrrole polyamide derivatives synthesized in the present study. The preliminary biological results described here constitute an additional example of the effect of lipid modification for biologically active compounds.

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded by a Varian Mercury 300 or VXR 500 using $CDCl_3$, and ESI MS data were recorded by API III (Perkin Elmer) by direct infusion, using a mixture of $THF/CH_3OH/H_2O$ (15:4:1) with 0.1% HCOOH or 0.1% $HCOO^-NH_4^+$ as a solvent in the positive mode.

Synthesis of N-linoleoylpropane-1,3-diamine (7). To a solution of linoleic acid (1.89 g, 6.75 mmol) in ethanol-free chloroform (25 ml) were added HOBt (0.95 g, 7.0 mmol) and DIC (0.84 g, 7.0 mmol), and the solution was stirred at r.t. overnight. A solution of 1,3-propanediamine (1.0 g, 13.5 mmol) in ethanol-free chloroform (7 ml) was added dropwise to the reaction mixture which was stirred at r.t. overnight. After evaporating the solvent under reduced pressure, the residue was chromatographed on silica gel, eluting with a mixture of cloroform/methanol/aq.NH_3 (80:20:5) to afford half amide **7**. $R_f = 0.65$ ($CHCl_3$:CH_3OH:NH_3aq, 80:20:5). ^{1}H-NMR ($CDCl_3$)δ(ppm): 0.78 (3H, t, $J = 7.8$, $-CH_2-CH_3$), 1.16 (14H, m, $-(CH_2)_4-CH_2-(CH{=}CH-CH_2)_2-(CH_2)_3-CH_3$), 1.50 (2H, m, $-C(O)-CH_2-CH_2-$), 1.60 (2H, d, $J = 6.6$ Hz, $-NH-CH_2-CH_2-CH_2-NH-$), 2.00 (4H, m, $-CH_2-CH{=}CH-CH_2-CH{=}CH-CH_2-$), 2.08 (2H, t, $J = 7.8$, $-C(O)-CH_2-$), 2.65 (2H, d, $J = 6.9$ Hz, $-CH_2-NH_2$), 2.75 (2H, t, $J = 6.5$, $=CH-CH_2-CH=$), 3.16 (2H, d, $J = 6.3$ Hz, $-NH-CH_2-$), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (4H, m, $-(CH{=}CH-CH_2)_2-$), 6.13 (1H, m, $-CH{=}CH-CH=$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, $(M + H^+)$ 337.2; $C_{21}H_{40}N_2O$ requires 336.6.

Synthesis of N-icosapentaenoylpropane-1,3-diamine (8). This intermediate was prepared under the same conditions as those used for the preparation of **7**. $R_f = 0.67$ ($CHCl_3$:CH_3OH: NH_3aq, 80:20:5). ^{1}H-NMR ($CDCl_3$): δ(ppm): 0.95 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.60 (2H, d, $J = 6.6$ Hz, $-NH-CH_2-CH_2-CH_2-NH-$), 1.75 (2H, m, $-C(O)-CH_2-CH_2-$), 2.10 (4H, m, $-CH_2-CH{=}CH-$, $-CH_2CH_3$), 2.30 (2H, t, $J = 8.0$, $-C(O)-CH_2-$), 2.65 (2H, d, $J = 6.9$ Hz, $-CH_2-NH_2$), 2.80 (8H, m, $-(CH{=}CH-CH_2)_4-$), 3.16 (2H, d, $J = 6.3$ Hz, $-NH-CH_2-$), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (10H, m, $-(CH{=}CH-CH_2)_5-$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, $(M + H^+)$ 359.2; $C_{23}H_{38}N_2O$ requires 358.6.

Synthesis of the stearoyl derivative (9). To a solution of **2** (55.2 mg, 0.15 mmol) and HOBt (24.4 mg, 0.18 mmol) in DMF (3 ml) was added DIC (22.2 mg, 0.18 mmol). The reaction mixture was stirred at r.t. for 24 h under N_2. Amide **6** (45.6 mg, 0.15 mmol) and distilled ethanol-free chloroform (3 ml) were added to this reaction mixture which was stirred at 50 °C for 24 h under N_2. The solvent was evaporated under reduced pressure. The resulting residue was chromatographed in a silica gel column ($CHCl_3$/MeOH, 95:5) to give final compound **9**. $R_f = 0.57$ ($CHCl_3$:CH_3OH, 95:5). ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.80 (3H, t, $J = 7.0$, $-CH_2-CH_3$), 1.30 (28H, m, $-(CH_2)_{14}-CH_3$), 1.65 (4H, m, $-C(O)-CH_2-CH_2-$, $-NH-CH_2-CH_2-CH_2-NH-$), 2.20 (2H, t, $J = 8.0$, $-C(O)-CH_2-$), 3.35 (4H, m, $-NH-CH_2-CH_2-CH_2-NH-$), 3.90–4.00 (9H, s, 3 × N–CH_3), 6.15 (1H, m, $-CH{=}CH-CH=$), 6.60–6.90 (6H, m, protons on the pyrrole rings) ESI MS m/z: found, $(M + H^+)$ 692.4; $C_{39}H_{61}N_7O_4$ requires 691.5.

Synthesis of the linoleoyl derivative (10). This product was prepared under the same conditions as those used

for the synthesis of **9**. TLC (Silica gel) $R_f = 0.38$ ($CHCl_3$:CH_3OH, 95:5); ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.85 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.30 (14H, m, $-(CH_2)_4-CH_2-(CH=CH-CH_2)_2-(CH_2)_3-CH_3$), 1.60 (4H, m, $-C(O)-CH_2-CH_2-$, $-NH-CH_2-CH_2-CH_2-NH-$), 2.00 (4H, q, $J = 7.5$, $-CH_2-CH=CH-CH_2-CH=CH-CH_2-$), 2.20 (2H, t, $J = 8.0$, $-C(O)-CH_2-$), 2.75 (2H, t, $J = 6.5$, $=CH-CH_2-CH=$), 3.35 (4H, m, $-NH-CH_2-CH_2-CH_2-NH-$), 3.90–4.00 (9H, s, $3 \times N-CH_3$), 5.35 (4H, m, $-(CH=CH-CH_2)_2-$), 6.60–6.90 (6H, m, protons on the pyrrole rings); ESI MS m/z: found, $(M + H)^+$ 688.5; $C_{39}H_{57}N_7O_4$ requires 687.5.

*Synthesis of the icosapentaenoyl derivative (**11**).* This product was prepared under the same conditions as those used for the synthesis of **9**. $R_f = 0.32$ ($CHCl_3$:CH_3OH, 95:5). ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.95 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.75 (4H, m, $-C(O)-CH_2-CH_2-$, $-NH-CH_2-CH_2-CH_2-NH-$), 2.10 (4H, m, $-CH_2-CH_2-CH=CH-$, $-CH_2-CH_3$), 2.30 (2H, t, $J = 8.0$, $-C(O)-CH_2-$), 2.80 (8H, m, $-(CH=CH-CH_2-CH=CH)_4$), 3.40 (4H, m, $-NH-CH_2-CH_2-CH_2-NH-$), 3.90–4.00 (9H, , s, $3 \times N-CH_3$), 5.35 (10H, m, $-(CH=CH-CH_2)_5-$), 6.13 (1H, m, $-CH=CH-CH=$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, $(M + H)^+$ 710.4; $C_{41}H_{55}N_7O_4$ requires 709.4.

*Synthesis of methyl γ-aminobutylate derivative of pyrrole polyamide (**12**).* A mixture of intermediate acid **2**, (312 mg, 0.8 mmol), HOBt (137 mg, 1.03 mmol) and DIC (130 mg, 1.03 mmol) in DMF (0.68 ml) was stirred at r.t. for 24 h. To this solution were added methyl 4-aminobutylate hydrochloride (130 mg, 0.85 mmol) and DIEA (260 µl, 1.49 mmol), and stirred at r.t. for further 24 h. After an addition of deionized water (10 ml), the product was extracted with chloroform. The product was purified by silica gel chromatography (hexane/ethyl acetate, 3:7) affording an unstable oil. TLC(Silica gel) $R_f = 0.3$ (Hexane:EtOAc, 2:8); ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.91 (2H, m, $-NH-CH_2-CH_2-$), 2.40 (2H, m, $-NH-CH_2-CH_2-CH_2-CO$), 3.39 (2H, m, $-NH-CH_2-$), 3.80 (3H, s, $O-CH_3$), 3.78–4.00 (9H, s, $3 \times N-CH_3$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: Found, $(M + NH_4)^+$ 486.3; $C_{23}H_{28}N_6O_5$ requires 486.0.

*Synthesis of γ-aminobutylate derivative of the pyrrole polyamide (**13**).* A solution of the ester **11** (200 mg, 0.43 mmol) and 2*N*-NaOH (10 ml) in methanol (10 ml) was stirred at 60 °C for 12 h. After removing the solvent under reduced pressure, the residue was acidified with 1*N*-HCl, and the acid was extracted with a mixture of chloroform/methanol (2:1). Silica gel chromatography (chloroform/methanol, 9:1 → 5:5) afforded acid **13** as an unstable oil which was used as such for the next reaction.

*Synthesis of the phospholipid derivative (**14**).* To a solution of **13** (100 mg, 0.22 mmol) and HOBt (34.4 mg, 0.26 mmol) in a mixed solvent of $CHCl_3$ (1 ml) and DMF (1 ml) was added DIC (32.8 mg, 0.26 mmol). The reaction mixture was stirred at r.t. for 24 h under N_2. Lyso-PC (136.2 mg, 0.26 mmol) and DIEA (100 µl) were added to this reaction mixture which was stirred at room temperature for 24 h under N_2. The solvent was evaporated under reduced pressure. The resulting residue was separated by silica gel column chromatography ($CHCl_3$/MeOH, 6:4) monitored by preparative TLC ($CHCl_3$/MeOH/NH_3aq. 65:35:5) to yield final compound **13** as an yellow oil. TLC (silica gel) $R_f = 0.6$ ($CHCl_3$:CH_3OH:NH_3aq, 60:30:5); ^{1}H-NMR ($CDCl_3$:CD_3OD, 8:2) δ(ppm): 0.80 (3H, t, $J = 7.5$, $-CH_2-CH_3$), 1.05 (2H, m, $-CH_2-CH_3$), 1.20 (26H, m, $-(CH_2)_{13}-CH_2-CH_3$), 1.50 (2H, $-C(O)-CH_2-CH_2-$), 1.90 (2H, m, $-NH-CH_2-CH_2-$), 2.20 (2H, m, $-NH-CH_2-CH_2-CH_2-$), 2.40 (2H, m, $-CH_2-O-C(O)-CH_2-$), 2.90 (2H, m, $-NH-CH_2-$), 3.30 (2H, m, $-CH_2-N-(CH_3)_3$), 3.50 (9H, s, $-N-(CH_3)_3$), 3.70–3.90 (9H, s, $3 \times N-CH_3$ on the pyrrole rings), 4.00 (2H, m, $-CH-CH_2-O-P-$), 4.10 (2H, m, $-CH_2-CH_2-O-P-$), 4.20 (2H, m, $-CH-CH_2-O-C(O)-$), 5.10 (1H, m, $-CH_2-CH-CH_2-$), 6.00 (1H, m, $-CH=CH-CH=$), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS m/z: found, $(M + H)^+$ 960.6; $C_{48}H_{78}N_7O_{11}P$ requires 959.6.

In vitro cytotoxicity and anti-HIV assays were respectively conducted by the microplate method and magic-5 method reported by Otake *et al.*[19] and Kimpton *et al.*[20]

Microplate method. Sample solutions (100 µl) were sequentially diluted at 1:2 or 1:5 with an RPMI1640 medium containing 10% FCS in a 96-well plate. For the cytotoxicity experiment, 100 µl of cell a suspension of MT-4 cells (2×10^5/ml) in a stage of exponential growth was added to each well. For the anti-HIV experiment, MT-4 cells (2×10^6) were infected by the addition of a stock solution of HTLV-BIII to a concentration suitable as an infectious dose (100TCID50) to the tissue culture, which was incubated at 37 °C for 1 h. The cells were resuspended in 10 ml of the medium, and the suspension (100 µl) was added to all the wells in the 96-well plate. After incubating for 5 days, the cytotoxicity and cytopathic effect (CPE) were evaluated by counting the cells by optical microscopic observation.

Magic-5 method. Magic-5 cells (10^4 cells) per one well of a 96-well plate were cultured at 37 °C to the stage at which the cells were allowed to adhere to the plastic surface of the plate. After removing the culture medium, a sample solution of the pyrrole polyamide diluted 2 times with the medium was added, this being followed by the addition of HIV-1 Ba-L strain prepared to a concentration of 100–200 BFU/50 µl by using the medium containing DEAE-dextran. The cells were incubated at 37 °C for 48 h in a CO_2 incubator. After removing the medium, 1%-formaldehyde and 0.2%-glutaraldehyde in PBS were added, and the mixture incubated at r.t. for 5 min. After washing the cells, 4 mM-potassium ferrocyanide, 4 mM-potassium ferricyanide, 2 mM $MgCl_2$ and 400 mg/ml of X-gal were added, and the mixture incubated at 37 °C for 1 h. The staining solution was removed and the cells were washed. The

cells stained blue were counted by using optical microscopic observation. In this experiment, TAK-779 and AZT were used as controls for the anti-HIV activity.

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Neocrocin A: a Novel Crocetin Glycoside with a Unique System for Binding Sugars Isolated from Gardenia Yellow

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A novel crocetin glycosyl ester, neocrocin A (2), was isolated from gardenia yellow. The structure of 2 was elucidated as that of an all-*trans*-crocetin β-D-gentiobiosyl β-D-glucopyranosyl-(1→6)-D-2-deoxy-glucopyranos-2-yl diester based on chemical and spectral data. The findings provide evidence that the binding system of crocetin glycosides is not limited to the anomeric position.

Key words *Gardenia jasminoides*; gardenia yellow; neocrocin A; crocetin glycoside; crocin

Crocin (**1**)[2)] is a digentiobiosyl all-*trans*-crocetin (8,8′-diapocarotene-8,8′-dioic acid) ester that is the major yellow pigment in gardenia yellow and saffron, which are extracts of *Gardenia jasminoides* fruits and *Crocus sativus* stigmas, respectively.[3,4)] Gardenia yellow and saffron consist of many minor pigments as well as some relatively abundant pigments which have been characterized as all-*trans*- and 13-*cis*-crocetin monoglucosyl ester,[2)] diglucosyl ester,[2)] monogentiobiosyl ester,[2,5)] glucosyl gentiobiosyl ester[2)] and gentiobiosyl neapolitanosyl ester.[5)] However, the structures of the other minor pigments have so far remained uncertain, and the binding systems for sugars have not previously been confirmed by detailed spectroscopic investigations, such as NMR analysis, after isolation. Here we report on the isolation and structural elucidation of a novel crocetin glycoside, neocrocin A (**2**), which has a unique binding system for sugars, based on spectral data and chemical derivatization (Fig. 1).

Results and Discussion

Gardenia yellow extracted from dried gardenia fruits was fractionated on a Diaion HP-20 column. The 60—70% methanol eluate was then concentrated and the residue was loaded into a preparative LC-MS system,[6)] led to the isolation of crocin (**1**) and neocrocin A (**2**).

Neocrocin A (**2**) was isolated as a red amorphous powder. The molecular formula of **2** was established as $C_{44}H_{64}O_{24}$, which was as the same as that of **1**, according to HR-ESI-MS (*m/z* 999.3707, $[M+Na]^+$, Calcd 999.3685), and the IR spectrum and UV/Vis absorption were similar to those of **1**. All-*trans*-crocetin dimethyl ester (**3**) and D-glucose were obtained, respectively, after the methanolysis and hydrolysis[7)] of **2**. These observations implied that **2** had the same carotenoid moiety, all-*trans*-crocetin, as the chromophore group, but that the binding system for glucoses differed from that of **1**. The ^{1}H- and ^{13}C-NMR spectra of **1** showed simple subduple signals, because **1** had C_2 structural symmetry. The ^{1}H-NMR spectra of **2** were similar to, but more complex than, those of **1**. Based on this comparison, we predicted that the C_2 structural symmetry was disrupted in **2** by the different binding system for glucoses at each end of crocetin, and that equilibrated isomerization could occur readily in the NMR solvent. The ^{1}H-NMR spectrum of **2** revealed a crocetin moiety (δ_H 6.49—7.31), anomeric doublets of β configuration (δ_H 5.38 (d, J=7.8 Hz), 4.53 (d, J=6.9 Hz), 4.13 (d, J=7.8 Hz), 4.15 (d, J=7.8 Hz)) and an anomeric doublet of α configuration (δ_H 5.06 (d, J=3.6 Hz)). Two anomeric protons (δ_H 4.53, 5.06) were shifted to a high magnetic field, and two oxymethines on H-2 (δ_H 4.39 (dd, J=3.7, 10.1 Hz), 4.53 (t, J=6.9 Hz)) were shifted to a low magnetic field, in comparison to those of **1**. This observation indicated the existence of a free hydroxyl group at an anomeric position on the glycosyl groups. Furthermore, HMBC correlations were observed between the H-2 of the α-glucoside and β-glucoside (glucose C) at δ_H 5.06 and δ_H 4.53, and the carbonyl carbons on the crocetin moiety at δ_C 167.92 and δ_C 167.11, respectively. Based on these chemical and spectral data, **2** was determined to be an all-*trans*-crocetin β-D-gentiobiosyl β-D-glucopyranosyl-(1→6)-D-2-deoxy-glucopyranos-2-yl ester. The NMR spectral data for **1** and **2** are summarized in Table 1.

Furthermore, to confirm the binding system for the sugars of neocrocin A (**2**), we firstly carried out peracylation of **2**. However, the sufficient quantity of peracetylated **2** was not obtained for the structure determination, because **2** was unstable more than crocin (**1**). Hence, after peracetylation of gardenia yellow, we isolated peracetylated crocin (**1a**) and an isomer of neocrocin A (**2a**). The other isomer of peracetylated **2** could not be isolated using preparative LC-MS because of overlapping with the peak of **1a**. The HR-ESI-MS spectra indicated that the molecular formula of both **1a** and **2a** was $C_{72}H_{92}O_{38}$. To compare the ^{1}H- and ^{13}C-NMR data between **2a** and **1a**, detailed 2D-NMR experiments were performed and the coupling constants were assigned using 1D-TOCSY. The chemical shifts and coupling constants of glu-

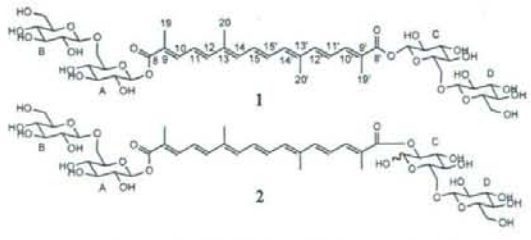

Fig. 1. Chemical Structures of Crocin (**1**) and Neocrocin A (**2**)

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Table 1. ^1H (800 MHz)- and ^{13}C (200 Mz)-NMR Spectral Data[a] of Crocin (**1**) and Neocrocin A (**2**) (in DMSO-d_6/D_2O (9 : 1))

Crocin (**1**)[b]			Neocrocin A (**2**)[b]											
			α isomer						β isomer					
No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C	No.	δ_H	δ_C
8,8'		166.80	8		166.72	8'		167.92	8		166.72	8'		167.11
9, 9'		125.70	9		125.77	9'		126.63	9		125.77	9'		127.08
10, 10'	7.31 (d, 11.0)	140.50	10	7.31 (d, 11.4)	140.44	10'	7.27 (d, 11.0)	139.42	10	7.31 (d, 11.4)	140.44	10'	7.20 (d, 11.0)	138.80
11, 11'	6.62 (br t, 14.7)	124.40	11	6.63 (br t, 11.7)	124.35	11'	6.62 (br t, 13.1)	124.52	11	6.63 (br t, 11.7)	124.35	11'	6.62 (br t, 13.1)	124.52
12, 12'	6.77 (d, 14.7)	145.20	12	6.78 (d, 14.6)	145.16	12'	6.72 (d, 15.1)	144.40	12	6.78 (d, 14.6)	145.16	12'	6.72 (d, 15.1)	144.14
13, 13'		137.40	13		137.45	13'		137.35	13		137.45	13'		137.33
14, 14'	6.50 (br d, 7.8)	136.60	14	6.49 (br d, 8.2)	136.52	14'	6.49 (br d, 8.2)	136.15	14	6.49 (br d, 8.2)	136.52	14'	6.49 (br d, 8.2)	136.05
15, 15'	6.81 (br d, 7.8)	132.50	15	6.82 (br d, 8.2)	132.56	15'	6.82 (br d, 8.2)	132.32	15	6.82 (br d, 8.2)	132.56	15'	6.82 (br d, 8.2)	132.22
19, 19'	1.92 (s)	13.16	19	1.92—1.95[c]	13.08—13.48[c]	19'	1.92—1.95[c]	13.08—13.48[c]	19	1.92—1.95[c]	13.08—13.48[c]	19'	1.92—1.95[c]	13.08—13.48[c]
20, 20'	1.94 (s)	13.04	20			20'			20			20'		
Glucose A, C			Glucose A			Glucose C			Glucose A			Glucose C		
1	5.37 (d, 7.8)	95.00	1	5.38 (d, 7.8)	95.05	1	5.06 (d, 3.6)	89.58	1	5.38 (d, 7.8)	95.05	1	4.53 (d, 6.9)	94.80
2	3.18 (t, 7.8)	72.78	2			2	4.39 (dd, 3.7, 10.1)	74.87	2			2	4.53 (t, 6.9)	75.87
3	3.23 (t, 8.0)	76.51	3			3			3			3		
4	3.20 (t, 8.0)	69.57	4	2.90—4.10[c]	68.00—77.00[c]	4	2.90—4.10[c]	68.00—77.00[c]	4	2.90—4.10[c]	68.00—77.00[c]	4	2.90—4.10[c]	68.00—77.00[c]
5	3.38 (m)	76.70	5			5			5			5		
6	3.54 (m)	68.36	6			6			6			6		
	3.94 (d, 10.1)													
Glucose B, D			Glucose B			Glucose D			Glucose B			Glucose D		
1	4.13 (d, 7.8)	103.49	1	4.13 (d, 7.8)	103.60	1	4.13 (d, 7.8)	103.70	1	4.13 (d, 7.8)	103.60	1	4.15 (d, 7.8)	103.70
2	2.91 (t, 8.0)	73.88	2			2			2			2		
3	3.08 (t, 8.0)	77.00	3			3			3			3		
4	2.99 (t, 8.0)	70.37	4	2.90—4.10[c]	61.00—77.00[c]	4	2.90—4.10[c]	61.00—77.00[c]	4	2.90—4.10[c]	61.00—77.00[c]	4	2.92—4.10[c]	61.00—77.00[c]
5	3.01 (m)	77.23	5			5			5			5		
6	3.38 (m)	61.38	6			6			6			6		
	3.60 (d, 10.5)													

a) Chemical shifts are in ppm and coupling constants in Hz are in parentheses. *b*) Assignments were made using 1D-TOCSY, COSY, HMQC and HMBC. *c*) Signals were overlapped and could not be assigned.