POTENT NON-PROTONOPHORE UNCOUPLERS ACTING ON NATURAL AND ARTIFICIAL MEMBRANES

JEAN-MARC ROUTABOUL, GEORGES TAILLANDIER,* PATRICK RAVANEL† and MICHEL TISSUT

Laboratoire de Physiologie Cellulaire Végétale (URA au CNRS 576), Université Joseph Fourier-Grenoble I, B.P. 53 X, 38041 Grenoble Cédex, France; †Laboratoire de Pharmacognosie, *Laboratoire de Chimie Organique, Université Joseph Fourier-Grenoble I, Domaine de la Merci, 38700 La Tronche, France

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Abstract—The uncoupling properties of 10 new symmetrical phenylureas, including *N,N'*-bis-(4-trifluoromethylphenyl)-urea, were investigated. Four compounds were shown to be powerful uncouplers. The result is a proton transfer across the organelle's membrane. These symmetrical phenylureas cannot be classified among the main class of uncouplers (acting through a protonophoric mechanism), due to their inability to exchange protons in a range of pHs between 2 and 9. The most potent uncouplers of the series induced permeabilization of artificial bilayer membranes (without protein) to both H^+ and monovalent cations (K^+ , Na^+) and did not increase the rate of diffusion of a small uncharged molecule (urea). The uncoupling properties for such molecules are due neither to their ability to induce conformational changes in membrane protein nor to their effect on phospholipid bilayer fluidity. They seem to change the regular organization of the polar lipid bilayer.

INTRODUCTION

In a previous paper [1], we described some biological properties of a symmetrical phenylurea: *N,N'*-bis-(4-trifluoromethylphenyl)-urea (USpCF₃). Among several interesting activities which were observed in isolated organelles, this compound was shown to be a highly potent uncoupler. The result of its action is a proton transfer through the biological membrane, leading to a collapse of the proton gradient required for ATP synthesis. The effectiveness of this compound is comparable to the protonophore reference uncoupler CCCP [2] or to platanetin, a natural lipophilic flavonoid extracted from plane-tree buds [3] but its mode of action is different: the symmetrical phenylurea being a non-protonophore. The physiological interest of this molecule led us to synthesize and study 10 derivatives of the same family including the *N,N'*-bis-(4-trifluoromethylphenyl)-urea. It seems to be of a great importance for the understanding of the uncoupling mechanism to know how such non-ionizable compounds can uncouple organelles. For this purpose, this paper reports some further investigations on artificial bilayer membranes in an attempt to gain insight into the molecular mechanism by which this molecule can induce a proton transfer across the biological membranes. Their mode of action could be to bring about a conformational change in the transmembrane proteins, leading to a H^+

permeability [4, 5] or a change in the organization or activity of the phospholipid bilayer [6].

RESULTS

Physical constants of the symmetrical phenylureas

The ability of the symmetrical phenylureas to ionize was studied. In order to do this, UV spectral shifts dependent on pH change (between 2 and 9) were looked for. No significant spectral variations were observed. This indicated that the symmetrical phenylureas, such as USpCF₃ [1], are unable to ionize. Similar results were obtained by a classical titrimetric method.

We also determined the lipophilicity of the compounds by three methods. The partition coefficient *P*, expressed by log *P* (a conventional measurement of the lipophilicity), (1) was measured spectrophotometrically through octanol water partitioning. The measurement of the lipophilicity by the partitioning method is neither an accurate nor an easy technique for such highly lipophilic compounds. Only three derivatives were measured by this method with sufficient accuracy; (2) was estimated by reversal phase TLC on silica-C18 (MeOH-H₂O, 17:3) and (3) was calculated theoretically by a method derived from ref. [7]. All the log *P* values are given in Table 1. The log *P* values differ depending on the method used, although there were good correlations between all these

*Author to whom correspondence should be addressed.

Table 1. Log *P* values for the symmetrical phenylureas studied

Substitution	Estimated by chromatography	Measured by spectrophotometry	Calculated
4-CF ₃	3.30	3.80	5.12
3,4-diCl	4.30		5.83
3-CF ₃	2.95		5.12
3-Cl	3.20		4.34
4-Cl	3.15	2.70	4.34
3-Me	2.60		3.90
3-OMe	2.35		3.02
Unsubstituted	2.25		2.86
4-OMe	1.95	2.70	3.02
4-Me	2.60		3.90

Table 2. Uncoupling activities (nmol mg⁻¹ protein) of the symmetrical phenylureas on isolated potato mitochondria measured by 50% uncoupling of the respiration rate with 6 mM succinate (+ATP 0.3 mM) as substrate, or by 100% swelling in a NH₄Cl or NH₄NO₃ isoosmotic medium

Substitution	Common name	<i>D</i> ₅₀ 50% uncoupling	100% swelling
4-CF ₃	USpCF ₃	5.9	11
3,4-diCl	USm,pCl	7.7	18
3-CF ₃	USmCF ₃	10	18
3-Cl	USmCl	14	18
4-Cl	USpCl	34	50
3-Me	USmMe	183	—
3-OMe	USmOMe	318	—
	USm,pH	818	—
Unsubstituted			
4-OMe	USpOMe	—	—
4-Me	USpMe	—	—

—No uncoupling activity even at saturating concentration.

values:

log *P* estimated by chromatography = 0.605 log *P* calculated + 0.353 (*n* = 10, *r* = 0.91)log *P* calculated = 2.096 log *P* measured spectrophotometrically - 2.730 (*n* = 3, *r* = 0.99)(*n*: number of values, *r*: regression coefficient).*Uncoupling activity of the symmetrical phenylureas on mitochondria*

Table 2 summarizes the uncoupling activities of the symmetrical phenylureas on isolated mitochondria. Four derivatives, *N,N'*-bis-(4-trifluoromethylphenyl)-urea (USpCF₃), *N,N'*-bis-(3,4-dichlorophenyl)-urea (USm,pCl), *N,N'*-bis-(3-trifluoromethylphenyl)-urea (USmCF₃), *N,N'*-bis-(3-chlorophenyl)-urea (USmCl) caused complete uncoupling of electron transfer in mitochondria oxidizing succinate at concentrations lower or equal to 23 nmol mg⁻¹ protein (23 nmol mg⁻¹ protein being the concentration required with CCCP to obtain complete uncoupling). The same results were obtained when succinate was replaced by α -ketoglutarate or mal-

ate. During oxidation of exogenous NADH, the high inhibitory activity of some of these products on electron transfer could rapidly mask their uncoupling activity.

Concurrent spectrophotometric measurement of mitochondrial swelling in NH₄Cl or NH₄NO₃ isoosmotic media [1, 8] confirmed the uncoupling activity demonstrated by polarography (Table 2). The same concentrations of these four powerful phenylureas increased in the same way the O₂ consumption with succinate or the mitochondrial swelling in isoosmotic media containing NH₄⁺ ions (complete uncoupling was given by the reference protonophore uncoupler CCCP). The result of the symmetrical phenylureas action is a proton transfer across the mitochondrial membrane. The other derivatives were weak uncouplers or were without effect (Table 2).

Table 3. Comparison of the uncoupling and inhibitory activities (nmol mg^{-1} chlorophyll) of the symmetrical phenylureas on thylakoids and class A chloroplasts

Common name	Class A chlor.	I_{50} 50% inhibition	Thylakoids D_{50} 50% uncoupling
	I_{50} 50% inhibition		
USpCF ₃	8.7	250	10.9
USm.pCl	17.4	125	17.4
USmCF ₃	26.1	162	21.7
USmCl	34.8	375	39.1
USpCl	—	—	174
USmMe	—	2500	869
USmOMe	—	3750	1522
USm.pH	—	4375	1739
USpOMe	—	—	4783
USpMe	—	—	—

—No uncoupling effect even at saturating concentration.

Fig. 1. Polarographic traces showing the uncoupling activity of USpCF₃ on *Acer* cells ($0.48 \text{ mg protein ml}^{-1}$). (1) Respiratory control trace; (2) respiratory control trace in the presence of CCCP ($10.4 \text{ nmol mg}^{-1}$ protein); (3) respiratory trace in the presence of USpCF₃. Numbers on the traces refer to nmol O_2 consumed $\text{min}^{-1} \text{ mg}^{-1}$ protein. KCN: $100 \mu\text{M}$.

ureas inhibiting electron transfer in thylakoids were acting at the level of photosystem II (PS II) and were inactive on photosystem I (PS I) (results not shown). These results suggested that uncoupling of the photophosphorylation process was directly responsible for the inhibition of the whole photosynthetic mechanism shown by O_2 evolution in class A chloroplasts.

Uncoupling activity of the symmetrical phenylureas, in situ, on a suspension culture of *Acer pseudoplatanus* cambial cells

A dense suspension of *Acer* cells was put into the reaction vessel of an oxygen electrode. The oxygen consumption of such a suspension, which reached $50 \text{ nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ protein, was greatly increased by $10.4 \text{ nmol CCCP mg}^{-1}$ protein and was fully suppressed by KCN $100 \mu\text{M}$ (Fig. 1). Under the same experimental conditions, 16.7 , 26.2 , 32.5 and $48.1 \text{ nmol mg}^{-1}$ protein of USpCF₃, USm.pCl, USmCF₃ and USmCl, respectively, induced the same increase of the cyanide-sensitive respiratory rate as that obtained with CCCP.

Uncoupling and inhibitory activity of the symmetrical phenylureas on thylakoids and chloroplasts

Table 3 summarizes the inhibitory and the uncoupling activities of symmetrical phenylureas on isolated thylakoids and on light-driven O_2 evolution in intact chloroplasts. For each of the four most active compounds, a similar concentration was required to inhibit O_2 evolution in class A chloroplasts and to uncouple the phosphorylation process in thylakoids. Moreover, the inhibition of electron transfer from H_2O to methylviologen in uncoupled thylakoids (in the presence of $5 \text{ mM NH}_4\text{Cl}$) occurred at much higher concentrations and this inhibition of electron transfer could not explain the inhibition of photosynthesis in intact chloroplasts. As for USpCF₃ [1], we demonstrated that all the symmetrical phenyl-

Permeabilization of artificial bilayer membrane to cations by the symmetrical phenylureas

Liposomes are usually impermeable to protons and this was the case for the soya lecithin liposomes used in this study. Hinkle's method [9] shows that the permeabilization of liposomes to cations can be obtained after addition of an uncoupler. We followed the electron transfer between an external hydrophilic reductor unable to penetrate into liposomes (sodium ascorbate) and a hydrophilic acceptor enclosed in liposomes (potassium ferricyanide) with a lipophilic electron carrier (tetramethyl-*p*-phenylenediamine: TMPD) which can easily cross the artificial membrane. The transmembrane H^+ gradient limits strictly the electron transfer across the membrane. The uncoupler inducing a H^+ permeabiliz-

ation, suppresses the transmembrane charge gradient, and consequently increases the ferricyanide reduction rate. USpCF_3 (25 μM) induced a 3.2-fold increase in the average reduction rate of the inner ferricyanide (Fig. 2) which rose from 0.059 to 0.18 $\Delta A \text{ min}^{-1}$ during the first 4 min. This increase corresponds to a rapid transfer of cations across the liposome membrane. Under the same experimental conditions, USm,pCl , USmCF_3 , USmCl and USpCl induced an increase in the reduction rate of 0.140, 0.122, 0.082, 0.045 $\Delta A \text{ min}^{-1}$, respectively. Other symmetrical phenylurea derivatives were without effect. For comparison, the protonophore reference uncoupler platanetin, at 25 μM , induced an increase in the ferricyanide reduction rate which was only 1.5-fold higher (0.065 $\Delta A \text{ min}^{-1}$) than that of the untreated sample.

Similar results were obtained when measuring the liposome swelling rate in isoosmotic NH_4Cl medium. Figure 2 shows that 25 μM of USpCF_3 induced an average swelling rate of 0.11 $\Delta A \text{ min}^{-1}$ during the first 4 min when the untreated sample remained unchanged. Under the same experimental conditions, USm,pCl , USmCF_3 , USmCl , USpCl and reference uncoupler platanetin induced an average rate of swelling of 0.085, 0.055, 0.030, 0.028, 0.065 $\Delta A \text{ min}^{-1}$, respectively. The other products of the series were ineffective.

Correlations were studied between the uncoupling activity on organelles and cells (D_{50} ; nmol mg^{-1}) and the induction of liposome H^+ permeabilization ($\Delta A \text{ min}^{-1}$). The best correlations were found with the permeabilization values obtained with the liposome swelling rate

Reference K^+ carrier, valinomycin, at 25 μM , induced an average rate of swelling of 0.055 $\Delta A \text{ min}^{-1}$.

Modification of the membrane fluidity

Liposomes spontaneously swell in a urea isoosmotic medium. This small molecule, without charge, diffuses slowly across the membrane inducing a change in the osmotic pressure and as a consequence an influx of water. Addition of USpCF_3 did not change the speed of penetration of urea suggesting that the symmetrical phenylurea did not modify the membrane fluidity. Even after a 15 min incubation period with the symmetrical phenylurea, the penetration rate of urea remained unchanged.

DISCUSSION

Several derivatives of the symmetrical phenylurea series studied here are among the most powerful uncouplers of phosphorylation processes. Due to their non-ionizability, these highly lipophilic compounds [1, 10] cannot transport H^+ directly as classical uncouplers do [2, 11–16].

Among the studied series, only *p* or *m* CF_3 , *p* or *m* Cl or *m,p* diCl derivatives are potent uncouplers. The other symmetrical phenylureas are almost ineffective as uncouplers. A very high lipophilicity seems to be required, in this series, to show potent uncoupling action ($\log P > 4.34$). In fact, a multiple linear regression correlation study between physico-chemical parameters and uncoupling activity in thylakoids and mitochondria gave interesting equations with $\log P$ or $\Sigma\sigma$ values ($\Sigma\sigma$ being highly correlated here with $\log P$; $r = 0.84$):

$$\log 1/D_{50} \text{ mitochondria} = 0.734 \log P + 1.856 \quad (n = 8, r = 0.938, s = 0.308, F = 43.55)$$

$$\log 1/D_{50} \text{ thylakoids} = 0.912 \log P + 1.441 \quad (n = 10, r = 0.886, s = 0.513, F = 29.16)$$

$$\log 1/D_{50} \text{ mitochondria} = 3.040 \Sigma\sigma + 4.185 \quad (n = 8, r = 0.920, s = 0.346, F = 33.10)$$

$$\log 1/D_{50} \text{ thylakoids} = 3.307 \Sigma\sigma + 4.637 \quad (n = 10, r = 0.963, s = 0.297, F = 103.27)$$

measurement (Fig. 3). The high degree of correlation found with the best equation ($r = 0.99$) suggests an identical mode of action of the studied compounds on liposomes, organelles and on *Acer* cells.

In marked contrast, none of the parameters used to express the shape of the molecule (length, width, volume, etc.) was able to give valuable equations.

$$D_{50} \text{ mitochondria} = 0.299 \text{ H}^+ \text{ permeabilization} + 3.297 \quad (n = 4, r = 0.985)$$

$$D_{50} \text{ thylakoids} = 0.109 \text{ H}^+ \text{ permeabilization} + 0.261 \quad (n = 4, r = 0.993)$$

$$D_{50} \text{ cells} = 0.775 \text{ H}^+ \text{ permeabilization} - 6.283 \quad (n = 4, r = 0.985)$$

Swelling experiments on liposomes were performed not only with NH_4Cl but also with NH_4NO_3 , KCl , NaCl , $\text{NH}_4\text{H}_2\text{PO}_4$ and $(\text{NH}_4)_2\text{SO}_4$ isoosmotic media (Fig. 4). For example, USpCF_3 was both an uncoupler (in NH_4NO_3 and NH_4Cl) and an ionophore able to induce permeabilization of liposome membranes to K^+ (0.1 $\Delta A \text{ min}^{-1}$) and Na^+ (0.065 $\Delta A \text{ min}^{-1}$). In contrast, platanetin, a potent uncoupler, is not an ionophore [10].

As the hypothesis of a direct H^+ transmembrane transport was ruled out (no pK_a in the biological pH range), the mode of action of these uncouplers could involve an effect either on the proteic part of the membranes or on the polar lipids. Liposomes, exclusively constituted of polar lipid bilayers, were used to demonstrate whether the polar lipids were involved in the

Fig. 2. Spectrophotometric traces showing the permeabilization of liposomes to protons. (1) Measured by the reduction of the potassium ferricyanide; (2) measured by the swelling in a NH_4Cl isoosmotic medium. U: uncoupler; ASC: sodium ascorbate 5 mM; TMPD: tetramethyl-*p*-phenylenediamine 25 μM . Numbers on traces refer to the average change in the absorbance observed for the first 4 min ($\Delta A \text{ min}^{-1}$).

Fig. 3. Comparison between liposome permeabilization to protons measured by swelling in isoosmotic medium and the uncoupling activity measured on *Acer* cells, potato mitochondria and on thylakoids for the four most potent symmetrical phenylureas.

uncoupling effect with the studied products. Our multilamellar vesicles were shown to be highly impervious to protons. The symmetric phenylureas found to be uncouplers in the organelles were also uncouplers in the liposomes, the concentrations needed in this latter case being five- to 10-times higher than those required for a

Fig. 4. Spectrophotometric swelling traces showing the permeabilization of a liposome isoosmotic suspension to protons and cations after addition of USpCF_3 . U: uncoupler. Numbers on traces refer to the average change in the absorbance observed for the first 4 min ($\Delta A \text{ min}^{-1}$).

complete uncoupling of organelles. The analogy of the uncoupling effect on isolated mitochondria, thylakoids, *Acer* cells and liposomes is sufficient to suggest that, in each case, the uncoupling process was the same.

The symmetrical phenylureas are thus potent non-ionophore uncouplers which paradoxically possess a mode of action both specific enough to induce permeabilization to some monovalent cations and not to other charged or uncharged molecules and unspecific and simple enough to act on different membranes including even the simplest one, the liposome. The only requirement seems to be the lipophilicity, even if the inefficiency of the *p*OMe and the *p*Me derivatives suggests that some steric exigencies could exist. Our results ruled out the hypothesis that the uncoupling effect could be due to a high affinity binding site on membrane protein components [4, 5].

The hypothesis of a detergent effect of these substances remains unlikely as their effect is quite different from classical compounds such as Triton X-100 [17-19]. The membrane permeability to H^+ , Na^+ and K^+ seems to originate from the binding of the phenylureas to the polar lipids, therefore perturbing the strict organization of the bilayer membranes. The possibility of an extra molecular link between the -NH and or the -CO group of the phenylurea with the phospholipid components has to be considered [6]. However, the permeability of the phospholipid bilayer to H^+ could also be the result of the

formation of stable phenylurea dimers inside the lipophilic membrane space.

EXPERIMENTAL

Synthesis. Symmetrical phenylureas were synthesized as previously described [1] by reaction of substituted phenylisocyanate with substituted aniline.

LogP measurement. Experimental values of $USpCF_3$, $USm.pH$ and $USm.Cl$ were obtained in an octanol/water system as previously described, after spectrophotometric measurements [1, 20]. LogP values of other products were estimated by reversal phase TLC (silica-C18, MeOH-H₂O, 17:3). The R_m values used to determine $-\log P$ were obtained using the equation: $R_m = \log(1/R_f - 1)$. The standard curve was constructed with 8 reference compounds (phenylcarbamate, metobromuron, metoxuron, diuron, naphthalene, chlortoluron, phenanthrene, bibenzyl). The correlation equation between the $-\log P$ values of the 8 standards [21, 22] and the measured R_m was: $\log P = 3.771 + 2.826 R_m$ ($r = 0.981$). LogP values were also calculated by a method derived from ref. [7], using the experimental $-\log P$ value given by ref. [21] for biphenylurea.

pKa measurement. Experimental values of pKa were looked for according to ref. [1].

Preparation of chloroplasts. Spinach (*Spinacia oleracea* L., Chenopodiaceae) leaves class A and C chloroplasts were obtained as previously described [23].

Preparation of mitochondria. Mitochondria from potato tubers (*Solanum tuberosum* L., Solanaceae) were prepared by methods previously described [24].

O₂ exchange measurements. O₂ exchange was followed polarographically at 25°C, using a Clark-type electrode system. For plant mitochondria, the reaction medium contained 0.3 M mannitol, 5 mM MgCl₂, 10 mM KCl, 10 mM Pi buffer, and 0.1% BSA. All the incubations were carried out at pH 7.2. For measuring photosynthetic O₂ exchange, the reaction medium contained 0.3 M mannitol, 50 mM MOPS (morpholinopropane sulphonic acid), 2 mM EDTA, pH 7.6. The release of O₂ by class A chloroplasts was measured after the addition of 0.5 mM NaHCO₃ and 0.1 mM Na₂HPO₄. In thylakoid membranes (23 µg chlorophyll ml⁻¹ medium), electron transfer was measured on uncoupled organelles (NH₄Cl, 5 mM), in the presence of the electron acceptor methylviologen (paraquat, 0.1 M), KCN (0.1 mM) was added at the beginning of the experiment, to inhibit any possible catalase activity.

Uncoupling test for thylakoids. Measurements of the uncoupling effect were made on light-driven electron flow through PS II → PS I. The uncoupling effect gave rise to an increase in the O₂ consumption rate. A 100% uncoupling effect was obtained when the addition of NH₄Cl (5 mM) did not induce further increases in O₂ consumption.

Uncoupling test for mitochondria. A suspension of intact mitochondria was energized. After a state III-state IV transition, the uncoupling effect of the product added at this stage corresponds to an increase in the oxidation

rate. A 100% uncoupling effect was obtained when the rate of O₂ was not further stimulated by the addition of CCCP (5 µM) or ADP (200 µM).

Mitochondrial swelling. Mitochondrial swelling was measured as previously described [20], by the apparent absorbance decrease at 540 nm. The reaction medium contained 100 mM NH₄NO₃ or NH₄Cl, 15 mM Tris-HCl, 0.1% BSA, pH 7.2. A rapid passive swelling was induced by uncouplers.

Protein and chlorophyll measurements. Protein contents were determined according to ref. [25] and chlorophyll concentration were measured as described in ref. [26].

Preparation of liposomes. Liposomes were prepared from Type II-s and III-s soybean L-α-phosphatidylcholine (Sigma) from methods derived from refs [6, 9]. Phosphatidylcholine (500 mg) was dissolved in 15 ml petrol. The soln was dried under partial vacuum, at room temp. in the dark in an attempt to form a uniform film on the surface of the flask. The film was further stirred, for 3 hr, at 25°C, with 10 ml of a dispersion soln (20 mM MOPS [morpholinopropane sulphonic acid], 1 mM EDTA and 0.6 M mannitol for swelling test or 0.2 M potassium ferricyanide, pH 7.5). The liposome suspension was then dialysed for 12 hr against 3-fold 500 ml 20 mM MOPS, 1 mM EDTA, 0.6 M mannitol.

Uncoupling tests for liposomes. Permeabilization to cations of a suspension of liposomes was followed by a method derived from ref. [9]. We followed the electron transfer induced between an hydrophilic reductant (sodium ascorbate) unable to cross the membrane and an acceptor (potassium ferricyanide) included inside the liposomes. A lipophilic electron carrier, tetramethyl-p-phenylene diamine (TMPD), transports the electrons across the membrane. The transmembrane charge gradient limits the transfer of electrons. The addition of an agent which induces a permeabilization to cations, suppresses the transmembrane charge gradient increasing the rate of reduction of ferricyanide. The reduction of the potassium ferricyanide was followed spectrophotometrically at 416 nm. Liposome suspension (50 µl) was diluted in 2 ml reaction medium (0.6 M mannitol, 1 mM EDTA, 20 mM MOPS, pH 7.5). The included ferricyanide absorbance value of this suspension was 1.9 at 416 nm in a 1 cm cuvette. After addition of sodium ascorbate (5 mM), tetraphenylboron (20 µM) and of uncoupler (dissolved in EtOH; final concn in the cuvette: 10⁻³), the ferricyanide reduction started with the addition of TMPD (25 µM).

Liposome swelling. Liposome swelling was measured with a technique derived from mitochondria swelling methods [8], by the apparent absorbance decrease at 540 nm. Liposome suspension (100 µl) was diluted in 2 ml of the reaction medium containing 0.3 M NH₄Cl or NH₄NO₃, 1 mM EDTA and 20 mM MOPS, pH 7.5. The absorbance at 540 nm was close to 0.9 in a 1 cm cuvette. A rapid passive swelling was induced by the addition of the uncoupler. The same method was used for the measurement of membrane permeabilization to cations with KCl, NaCl, MgCl₂ or CaCl₂ isoosmotic media.

Q.S.A.R. analysis. Correlations between the activity of the uncouplers on organelles and some parameters

describing their physical or chemical properties were calculated using a multilinear regressive analysis. The parameters used were $\log P$ [7, 21], $\Sigma\sigma$ [21], $^1\chi^v$ (molecular connectivity [27]), ΔRM (molecular refraction [21]), L and B (length and width of the substituents [28]). r : regression coefficient, s : standard deviation of the regression, t : Student's test value, F : F test value.

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