*Accelerated Publications*

# Effect of Chemically Well-Defined Sphingosine and Its *N*-Methyl Derivatives on Protein Kinase C and src Kinase Activities†

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ABSTRACT: In view of the possible effects of the sphingoid base on protein kinases, and the fact that the sphingoid bases used in previous studies were not chemically well-defined, we have studied the effects of chemically well-defined sphingosines and their derivatives on kinase activity. Both (4*E*)-D- and (4*E*)-L-*erythro*-sphingenine showed a weak inhibitory effect, and (4*E*)-L-*threo*-sphingenine had a moderate inhibitory effect. In contrast, (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine and the sphingosine preparation from a commercial source showed a strong inhibitory effect on PK-C in A431 cells as well as on purified PK-C. Synthetic (4*E*)-D-*erythro*-sphingenine and several samples of natural sphingosine inhibited v-src or c-src tyrosine kinase activity measured with polyglutamate–tyrosine (4:1) as substrate. N-Acetylated or N-methylated sphingosines did not inhibit src kinase activity, but rather produced a consistent 1.5–2-fold stimulation of such activity.

Sphingosine and sphingoid bases have been shown to be modulators of PK-C[1] (Hannun & Bell, 1987) and epidermal growth factor receptor associated tyrosine kinase (Northwood & Davis, 1988; Davis et al., 1988; Faucher et al., 1988) and implicated as having a role in modulating transmembrane signal transduction [for a review, see Hannun and Bell (1989)]. In these studies, however, the sphingosine used was from a commercial source and was obtained through classical methanolysis of cerebroside or sphingomyelin, which involves a variety of methylated derivatives, erythro/threo and cis/trans conversion, and unavoidable oxidation through unknown mechanisms and structures (Weiss, 1964; Taketomi & Kawamura, 1972; Hara & Taketomi, 1986). It was previously observed (Abdel-Ghany et al., 1989) that sphingosine preparations from Sigma Chemical Co. were much more potent inhibitors of PK-C than were pure synthetic sphingosines. In view of the implicated effects of sphingoid bases, we studied the effect of chemically well-defined synthetic sphingosines and their *N*-methyl and *N*-acetyl derivatives as compared to the effects of the sphingoid base fraction from a commercial source on PK-C and src kinase activity.

## Materials and Methods

*Sphingosine Preparations.* The chemically well-defined sphingoid bases (4*E*)-D-(+)-*erythro*-sphingenine, (4*E*)-L-(−)-*erythro*-sphingenine, (4*E*)-L-(−)-*threo*-sphingenine, and L-(−)-*threo*-sphinganine (see Figure 1) were synthesized from D-glucose as previously described (Koike et al., 1984, 1985, 1986). Various sphingosine preparations from cerebroside and sphingomyelin, and synthetic random polyglutamate–tyrosine (4:1), were purchased from Sigma Chemical Co. (St. Louis, MO). (4*E*)-*N*-Monomethyl-D-*erythro*-sphingenine (**5** in Figure 2) was synthesized from (4*E*)-D-*erythro*-sphingenine by successive N-tert-butyloxycarbonylation (di-*tert*-butyl dicarbonate–$NaHCO_3$) (**1** in Figure 2), O-acetylation (acetic anhydride–pyridine) (**2** in Figure 2), N-methylation (methyl iodide–sodium hydride–dimethylformamide) (**3** in Figure 2), and deprotection through **4** in Figure 2 [(1) $NaOCH_3$, (2) $CF_3COOH$]. (4*E*)-*N*,*N*-Dimethyl-D-*erythro*-sphingenine and (4*E*)-*N*,*N*-dimethyl-L-*erythro*-sphingenine were prepared from (4*E*)-D-*erythro*- and (4*E*)-L-*erythro*-sphingenine, respectively, by reductive methylation (Means & Feeney, 1968). (4*E*)-*N*,*N*-Dimethylsphingenine was also derivatized from (4*E*)-*N*-monomethylsphingenine ($CH_3I$, $K_2CO_3$ in $CH_3OH$). The procedure for synthesis of *N*-methyl derivatives of (4*E*)-D-*erythro*-sphingenine is summarized in Figure 2. Compounds were purified by preparative thin-layer chromatography, and structures were verified by proton nuclear magnetic resonance and mass spectrometry (T. Toyokuni and B. Dean, unpublished results).

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PK-C, protein kinase C; TCA, trichloroacetic acid. A generic term "sphingosine" is used to designate a group of related long-chain aliphatic 2-amino-1,3-diols without regard to structural variation involving chain length, stereoisomers $C_1$–$C_3$ (D/L; threo/erythro), or double bond (cis/trans). The position and geometric isomer of the olefinic double bond are indicated as recommended by Fletcher et al. (1974); i.e., sphingenine having a 4,5 trans or cis double bond is designated respectively as 4*E* or 4*Z*. The term "sphingoid base" is used when the structure is not clearly defined, but is an aliphatic base.

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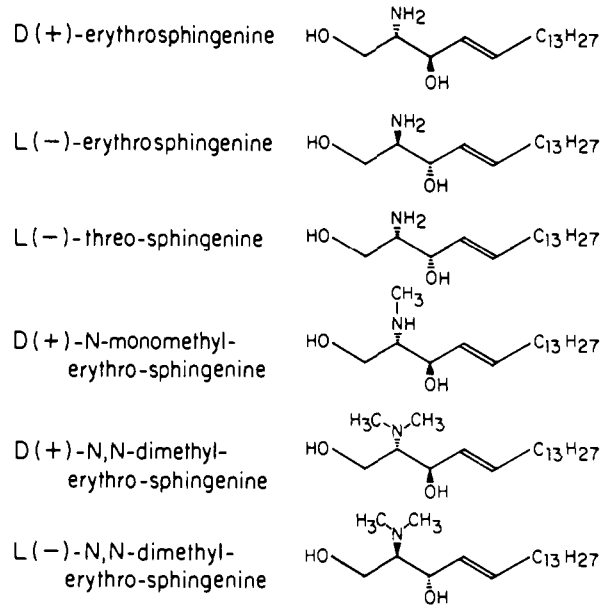


FIGURE 1: Structures of sphingosines and their derivatives used in this study. All samples were 4*E* isomeric forms.

*Determination of PK-C Activity of A431 Cells.* A431 cells in culture were harvested and treated simultaneously for partial purification of PK-C by the method of Kreutter et al. (1987). Briefly, cells (from 50- to 150-cm diameter dishes) were suspended in 50 mL of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 0.15 units/mL aprotinine, and 0.25 M sucrose and homogenized by 50 strokes at 4 °C in a Dounce homogenizer. The homogenate was ultracentrifuged at 100000*g* for 60 min, and the supernatant was purified on a DE52 column equilibrated with 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.5 mM EGTA (buffer B) and washed well with this buffer. The PK-C activity was eluted with buffer B containing 0.1 M NaCl. The activity in this fraction was 200–500 pmol of P $min^{-1}$ (mg of protein)$^{-1}$. The fraction, free of protein kinase A and other kinases, was aliquoted and kept at −80 °C.

The standard liposome method described by Kraft and Anderson (1983) was used with slight modification, and the effects of sphingosines and derivatives were compared under this system rather than a mixed micelle system.[2] Briefly, in conical tubes (1.5-mL content, Sarstedt), phosphatidylserine (5 μg/tube) and 1,2-diolein (0.05 μg/tube), with or without an appropriate quantity of sphingosines and derivatives, were added in organic solvent (ethanol or chloroform–methanol), and the mixture was evaporated under an $N_2$ stream. The lipid mixture was sonicated in 30 μL of 20 mM Tris-HCl (pH 7.5) for 30 min. The liposomes in the tube were supplemented with the reaction mixture, consisting of 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 400 μM EDTA, 50 μM EGTA, 500 μM $CaCl_2$, 200 μg/mL histone III-S, and 20 μM [$\gamma$-$^{32}$P]ATP (2 × $10^6$ cpm); the final volume was 90 μL. The reaction was initiated by addition of 10 μL of the PK-C fraction (containing 1–2 μg of protein) prepared as described above, and the reaction mixture was incubated for 10 min at 30 °C. The reaction was terminated by addition of 1 mL of 25% TCA with 200 μL of 1% bovine serum albumin in 1 mM ATP solution (pH 7.5). The precipitate was centrifuged, washed twice with 1 mL of 25% TCA, dissolved in 1 mL of 1 M NaOH containing 0.1% deoxycholate with slight heating (80 °C for 10 min), and counted in a scintillation counter. The value without phosphatidylserine, 1,2-diolein, or $Ca^{2+}$ was used as a reference blank.

[2] The background value (i.e., that without phosphatidylserine/diolein/$Ca^{2+}$) varied from 30% to 70% of the value with phosphatidylserine/diolein/$Ca^{2+}$. Reliable data could not be obtained due to this extensive variation of the background value.

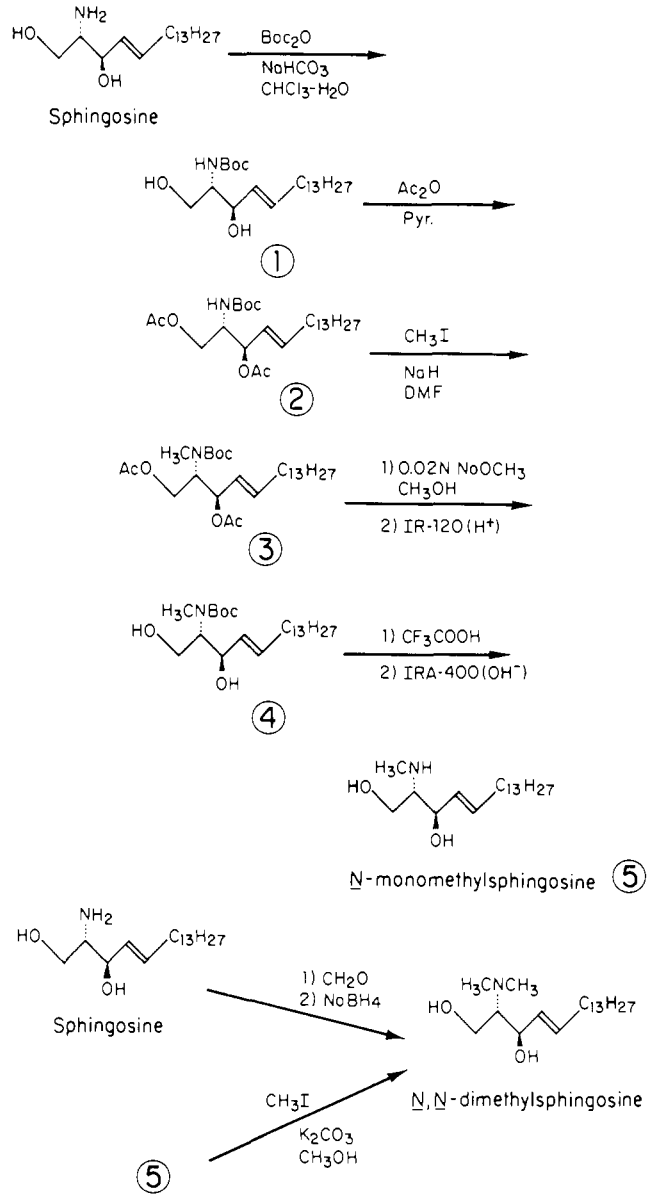


FIGURE 2: Preparation of (4*E*)-*N*-monomethyl-D-*erythro*-sphingenine and (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine.

*Purification and Assay of PK-C and src Kinases.* PK-C was purified from rabbit brain (Jaken & Kiley, 1987) and assayed by the method of Woodgett and Hunter (1987). SF-9 cells infected with *Baculovirus* containing either v-src or c-src expression vectors were generously supplied by Drs. R. Clark and E. C. O'Rourke of Cetus Corp. Purification was achieved with a monoclonal antibody donated by Dr. J. Brugge or by affinity chromatography using a synthetic random polymer containing tyrosine as described previously (Braun et al., 1984). Protein tyrosine kinase activity with polyglutamate–tyrosine as substrate was assayed as described (Braun et al., 1984).

## RESULTS

*Effects of Sphingosines on PK-C Activity under Standard Conditions.* The effects of sphingosines and their derivatives on PK-C activity of A431 cells are illustrated in Figure 3. Synthetic (4*E*)-D- or (4*E*)-L-*erythro*-sphingenines showed no

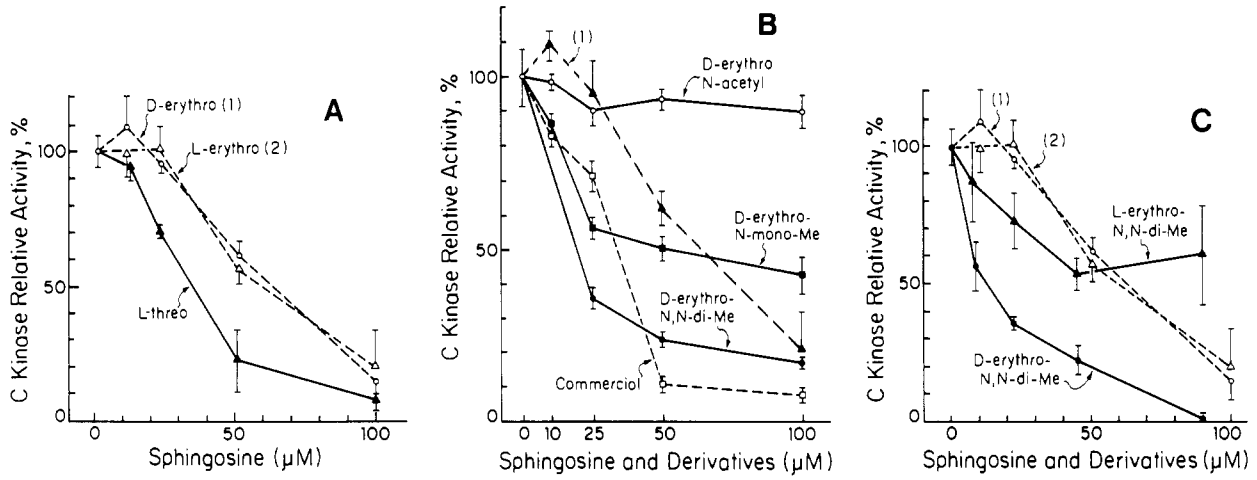


FIGURE 3: Effect of sphingosines on PK-C activity of A431 cells. Panel A: Comparison of the effects of (4*E*)-D-*erythro*-, (4*E*)-L-*erythro*-, and (4*E*)-L-*threo*-sphingenine. The 100% value of PK-C activity in the absence of sphingosine was 20 500 cpm per tube per 10 min, as measured by $^{32}P$ incorporation into histone III-S. Symbols: (○) (4*E*)-D-*erythro*-sphingenine; (△) (4*E*)-L-*erythro*-sphingenine; (▲) (4*E*)-L-*threo*-sphingenine. Vertical bars at the data points indicate standard deviation (SD). Note that (4*E*)-D-*erythro*- and (4*E*)-L-*erythro*-sphingenine showed no inhibitory activity at 25 μM, while (4*E*)-L-*threo*-sphingenine, the unnatural compound, showed moderate inhibitory activity. Panel B: Comparison of the effects of various N-substituted sphingosines, commercial sphingosines, and synthetic (4*E*)-D-*erythro*-sphingenine. The 100% value of PK-C activity in the absence of sphingosine was 19 200 cpm per tube per 10 min, as measured by $^{32}P$ incorporation into histone III-S. Symbols: (▲) (4*E*)-D-*erythro*-sphingenine; (●) (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine; (■) (4*E*)-*N*-monomethyl-D-*erythro*-sphingenine; (○) (4*E*)-*N*-acetyl-D-*erythro*-sphingenine; (□) commercial sphingosine preparation from Sigma Chemical Co. Vertical bars at the data points indicate SD. Note that the *N*,*N*-dimethyl derivative showed the strongest inhibitory activity, the *N*-monomethyl derivative showed weaker inhibitory activity, and the *N*-acetyl derivative had no inhibitory activity. Commercial sphingosine preparations, in comparison to (4*E*)-D-*erythro*-sphingenine, had a much stronger inhibitory activity comparable to that of the *N*,*N*-dimethyl derivative. Panel C: Comparison of the effects of (4*E*)-*N*,*N*-dimethylsphingenine with L and D configuration on PK-C activity. The 100% value of PK-C activity in the absence of sphingosine was 20 100 cpm per tube per 10 min, as measured by $^{32}P$ incorporation into histone III-S. Symbols: (●) (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine; (▲) (4*E*)-*N*,*N*-dimethyl-L-*erythro*-sphingenine; (○) (4*E*)-D-*erythro*-sphingenine; (△) (4*E*)-L-*erythro*-sphingenine. Vertical bars at the data points indicate SD. Note that there was a major difference in inhibitory activity when the (4*E*)-*N*,*N*-dimethyl-substituted derivatives of D- and L-*erythro*-sphingenine were used, whereas the parent sphingenines showed no such difference.

significant inhibitory effect at 25 μM. They did show an inhibitory effect at higher concentrations (50–100 μM), but there was no difference in effect between the D- and L-erythro isomers. However, synthetic (4*E*)-L-*threo*-sphingenine, the unnatural isomer, showed a weak but significant inhibitory effect at 25 μM (Figure 3A).

In contrast, (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine showed a strong inhibitory effect even at 10–25 μM concentration (Figure 3B,C), while the *N*-monomethyl derivative showed less inhibitory effect, particularly at low concentration (10–25 μM) (Figure 3B). (4*E*)-*N*,*N*-Dimethyl-L-*erythro*-sphingenine showed much less inhibitory effect than (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine (Figure 3C). Thus, the inhibitory effect of *N*,*N*-dimethyl derivatives of the naturally occurring D-erythro form is greater than that of the L-erythro form.

(4*E*)-*N*-Acetyl-D-*erythro*-sphingenine had no inhibitory effect (Figure 3C). Sulfatide, lactosylceramide, globoside, and lactosylsphingosine showed no effect at various concentrations tested (data not shown). Sphingosine preparations from Sigma showed variable inhibitory effects: some showed stronger effects than that of (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine, while others showed effects comparable to (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine. The inhibition curve of one sample showing moderate effect is shown in Figure 3B. A mixture of 3-*O*-methyl- and 5-*O*-methylsphingenine prepared from methanolysis products did not show any inhibitory effect at 100 μM concentration (data not shown).

As reported briefly previously (Abdel-Ghany et al., 1989), purified PK-C was inhibited by Sigma sphingosine and to a much lesser extent by pure sphingosine. These experiments were extended to a variety of synthetic and pure natural sphingosines. At 200 μM concentration, four synthetic sphingosines [(4*E*)-D-*erythro*-, (4*E*)-L-*erythro*-, and (4*E*)-L-*threo*-sphingenines and L-*threo*-sphinganine] produced an average of 22% inhibition, whereas Sigma sphingosine caused 92% inhibition.

*Effects of Sphingosines on src Kinase.* As shown in Table I, several preparations of natural or synthetic sphingosine produced inhibition of c-src or v-src kinase activity, as measured by phosphorylation of polyglutamate–tyrosine (4:1). At 330 μM, the inhibition was ≈60–70%; at 660 μM, ≈80–90%. Significant inhibition (20–35%) was observed at 75 μM. Sigma sphingosine showed a stronger inhibitory effect on both c-src and v-src kinases than did synthetic D-*erythro*-sphingenine in two separate experiments (Table I). Interestingly, N-modified sphingosine (i.e., N-acetylated sphingosine in this case) did not inhibit kinase activity, but rather produced a consistent ≈1.5-fold stimulation at either 330 or 660 μM. The stimulatory effect was even more striking with (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine (Table I, experiment II).

## Discussion

Hannun and Bell (1987, 1989) hypothesized that (i) sphingosine, the common component of all sphingolipids, has a strong inhibitory effect on PK-C activity, whereas diacylglycerol has a promoting effect; and (ii) positive and negative control of transmembrane signals is achieved through modulation of PK-C by a pair of lipid degradation products, one derived from phosphoglycerolipid and the other from sphingolipid.

Sphingosine preparations used in these previous studies were commercial products. They were prepared from sphingolipids by methanolysis followed by solvent partition and chromatography (Gaver & Sweeley, 1965) and are known to be mixtures of various derivatives induced during methanolysis (Weiss, 1964; Sambasivarao & McCluer, 1964; Taketomi & Kawamura, 1972; Hara & Taketomi, 1986). The effect of

Table I: Effect of Sphingosines on src Protein Tyrosine Kinase[a]

| addition | concn ($\mu$M) | c-src kinase | | v-src kinase | |
|---|---|---|---|---|---|
| | | cpm | % inhibition | cpm | % inhibition |
| experiment I | | | | | |
| none | | 20 300 | | 13 500 | |
| 2% ethanol | | 19 700 | <5 | 12 500 | <5 |
| D-*erythro*-sphingenine, synthetic | 330 | 6 500 | 66 | 5 500 | 55 |
| | 660 | 4 500 | 78 | 3 000 | 76 |
| sphingosine (from cerebroside) | 330 | 3 800 | 81 | 5 600 | 54 |
| | 660 | 1 900 | 90 | 3 000 | 76 |
| *N*-acetyl-D-*erythro*-sphingenine | 330 | 32 100 | *b* | 16 200 | *c* |
| experiment II | | | | | |
| none | | 22 000 | | 8 300 | |
| 2% ethanol | | 21 000 | <5 | 8 400 | <5 |
| D-*erythro*-sphingenine, synthetic | 330 | 9 000 | 59 | 2 300 | 72 |
| sphingosine (from cerebroside) | 330 | 3 400 | 85 | 1 000 | 88 |
| *N*-monomethyl-D-*erythro*-sphingenine | 330 | 12 000 | 45 | 3 200 | 61 |
| *N*,*N*-dimethyl-D-*erythro*-sphingenine | 330 | 40 000 | *d* | 12 200 | *e* |

[a] The assay mixture contained, in a final volume of 50 $\mu$L, 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 $\mu$g of polyglutamate–tyrosine (4:1), and 2 $\mu$g of c-src or 2.6 $\mu$g of v-src protein. After addition of ethanol or the indicated sphingosines (in ethanol), the reaction was initiated by addition of 10 $\mu$M [$\gamma$-$^{32}$P]ATP ($\approx$4000 cpm/pmol), followed by 10-min incubation at room temperature. Filter paper assays were performed as previously described (Abdel-Ghany et al., 1989). All sphingenines used were 4*E* isomeric forms. [b] 38–45% stimulation. [c] 22–25% stimulation. [d] 82% stimulation. [e] 47% stimulation.

the sphingoid base on protein kinases should be tested with chemically well-defined sphingosine, which can be prepared only through chemical synthesis (Koike et al., 1984, 1985, 1986). Another problem is the assay method used to observe the stoichiometry of an enzyme and its inhibitor. Since the use of a mixed micellar solution of lipids in the presence of a high concentration (0.3%) of detergent (e.g., Triton X-100) was emphasized in order to obtain stoichiometry of the effect of the sphingoid base on PK-C in previous studies (Hannun & Bell, 1987, 1989), we initially tried conditions similar to those described by these authors. However, we found that the results were extremely variable, making it difficult to observe effects of added lipids in mixed micellar conditions.[2]

For studies on the physiological effects of lipid modulators, it is highly desirable to use "standard conditions" in which conformation and organization of membrane-bound enzymes are as close as possible to the natural state. In the present study, using such standard conditions, we observed highly reproducible inhibitory effects of synthetic (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine and commercial sphingenine preparations on PK-C activity. In contrast, synthetic (4*E*)-D-*erythro*- or (4*E*)-L-*erythro*-sphingenine had much weaker effects on PK-C activity, while (4*E*)-L-*threo*-sphingenine showed a moderate inhibitory effect. Thus, the inhibitory effect on PK-C activity by sphingoid bases does not reflect naturally occurring substrate stereospecificity. The absence of stereospecific effect by sphingoid bases on PK-C activity poses the fundamental question of whether free sphingoid bases play a significant physiological role in modulation of PK-C function. If they do have such a role under natural physiological conditions, the active component may not be the natural form of (4*E*)-D-*erythro*-sphingenine, but rather an *N*-methyl or some other unidentified derivative. In fact, (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine had a much stronger effect than (4*E*)-D-*erythro*-sphingenine.

We have shown that pure (4*E*)-D-*erythro*-sphingenine inhibits the protein kinase activity of both v-src and c-src, although the Sigma sphingosine preparation showed stronger inhibitory effect in two separate experiments. It is likely that the Sigma preparation has an unidentified component with stronger inhibitory effect than (4*E*)-D-*erythro*-sphingenine (which represents the natural configuration). We showed previously (Abdel-Ghany et al., 1989) that protein kinase P, a casein kinase, was stimulated by pure (4*E*)-D-*erythro*-sphingenine and to a lesser extent by Sigma sphingosine.

The effective doses for sphingosines and their derivatives on src kinases were five to ten times higher than those for PK-C, although the specificity of the sphingosine effect on both src kinases and PK-C was quite clear. The susceptibility, but not specificity, of protein kinase activity to biomodulatory lipids depends greatly on the environmental conditions of the kinase in question. Under the conditions of the present study, susceptibility of src kinase to sphingosines was lower than that of PK-C. However, this does not imply that src kinase, compared with PK-C, is generally less susceptible to sphingoid bases. The in vitro effective concentration of bioactive lipids is generally very different from the actual concentration of the same lipids in vivo. For example, the effective doses of diacylglycerol and the sphingoid base are both in the range of 100–200 $\mu$M concentration (Hannun et al., 1986), while both lipids are found in physiological concentrations of only 10–50 pmol/$10^7$ cells (Wilson et al., 1988). Thus, compartmentalization of bioactive lipids in target structures may account for much of the observed differences between in vitro effective doses and in vivo cellular concentrations of these lipid modulators.

Interestingly, N-substituted sphingosines [i.e., (4*E*)-*N*-acetyl-D-*erythro*-sphingenine and (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine] were not inhibitory, but rather enhanced both c-src and v-src kinase activity (Table I). The stimulatory effect of (4*E*)-*N*,*N*-dimethyl-D-*erythro*-sphingenine on src kinase was particularly remarkable, in contrast to the strong inhibitory effect of the same compound on PK-C. This compound is characterized by the presence of two sterically unique hydrophobic $CH_3$ groups which cause an electron-donor effect. Since biosynthesis of this compound has been observed in some cells and tissues (Y. Igarashi and S. Hakomori, unpublished results), it may be a naturally occurring modulator of various kinases. In view of the multiple and variable effects of sphingosines and their derivatives on protein kinases, caution must be recommended in interpretation of the biological significance of these substances.

## Added in Proof

Since this paper was submitted and accepted for publication, another paper describing the effect of synthetic sphingosines with different stereospecific structures and chain lengths on protein kinase C was published (Merrill et al., 1989). Their

data indicate no difference in the inhibitory effects between different stereoisomers. A weaker inhibitory activity was observed with sphingosines having shorter aliphatic chains. Inconsistency between the results of this paper and theirs may depend on the assay conditions.


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# Evidence That a Major Determinant for the Identity of a Transfer RNA Is Conserved in Evolution†


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ABSTRACT: We observed recently that a single G3·U70 base pair in the amino acid acceptor stem of an *Escherichia coli* alanine tRNA is a major determinant for its identity. Inspection of tRNA sequences shows that G3·U70 is unique to alanine in *E. coli* and is present in eucaryotic cytoplasmic alanine tRNAs. We show here that single nucleotide changes of G3·U70 to A3·U70 or to G3·C70 eliminate in vitro aminoacylation of an insect and of a human alanine tRNA by the respective homologous synthetase. Compared to the influence of G3·U70, other sequence variations in $tRNA^{Ala}$ have a relatively small effect on aminoacylation by the insect and human enzymes. In addition, while these eucaryotic tRNAs have nucleotide differences from *E. coli* alanine tRNA, they are heterologously charged only with alanine when expressed in *E. coli*. The results indicate a functional role for G3·U70 that is conserved in evolution. They also suggest that the sequence differences between *E. coli* and the eucaryotic alanine tRNAs at sites other than the conserved G3·U70 do not create major determinants for recognition by any other bacterial enzyme.


The adaptor role of transfer RNAs is manifested through both their codon and amino acid specificities. The codon specificity is determined by the base pairing between the anticodons in tRNAs and the trinucleotide sequences of codons. The amino acid specificity is determined by unique sets of nucleotides that are important for contact with the cognate aminoacyl tRNA synthetases (Schulman & Abelson, 1988; Yarus, 1988; Schimmel, 1989). The conservation of the genetic code in evolution does not demand that the determinants for the identities of tRNAs be conserved. The only requirement is that the same trinucleotide sequences be assigned to each amino acid, and this in principle can be done with determinants for tRNA identity that are unique to each organism.

Early work demonstrated examples of cross-species aminoacylation, misacylation, and lack of aminoacylation (Jacobson, 1971). The results are not easy to interpret in the context of the question of conservation of determinants for tRNA identity. Failure of cross-species aminoacylation, for


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