The Inhibition of the Reverse Transcriptase of HIV-1 by the Natural Sulfoglycolipids from Cyanobacteria: Contribution of Different Moieties to Their High Potency

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Received December 31, 1997

The potent in vitro inhibition of the enzymatic activity of the human immunodeficiency virus-1 (HIV-1) reverse transcriptase (RT) by the lipophilic extracts of cyanobacteria[8] was primarily attributed to the sulfoquinovosylpranosyl lipids, compounds **1**−**4**. These sulfolipids inhibit efficiently and selectively only the DNA polymerase activity of HIV-1 RT (and not the ribonuclease H function) with 50% inhibitory concentration value (IC_{50}) as low as 24 nM exhibited by compound **1**. The novel natural compound **4**, in which two hydroxy groups on the sugar moiety are substituted by palmitoyl residues, exhibits a significant decrease in the maximal inhibition capacity. It is possible, therefore, that the contribution of acylated groups to the molecule at these positions interferes with inhibition, possibly, by steric hindrance. Both the sulfonic acid moiety and the fatty acid ester side chain have a substantial effect in potentiating the extent of inhibition. For one, the inhibitory effects of all the natural glycolipids tested (**5**−**8**) are markedly reduced, and the hydrolysis of the fatty acid side chain, as in derivative **9**, has substantially abolished the inhibition of HIV RT.

Reverse transcriptase (RT) of the human immunodeficiency virus (HIV) is a promising target for the chemotherapeutic treatment of AIDS. The crucial role of RT in the early stages of the HIV life-cycle contributes to the potency of inhibition, whereas specificity is determined by two reasons. First, its enzymatic properties are quite different from other cellular DNA polymerases, and, second, it is located in the cytoplasmatic compartment apart from the nucleic and mitochondrial DNA polymerases. RT is a multifunctional enzyme with two enzymatic activities—DNA polymerase and RNase H. Both functions are responsible for converting the viral genomic RNA into proviral double-stranded DNA.[1] This DNA is then transported from the cytoplasm into the nucleus, where it is subsequently integrated into cell DNA. Inhibition of each of the catalytic functions of RT interferes with the virus production in the host cell. In fact, the clinical potential of compounds that block the activity of HIV-1 RT has been demonstrated by the use of two groups of drugs, the nucleoside analogues and the so-called nonnucleoside RT inhibitors (NNRTIs).[2] The nucleoside analogues need first to be phosphorylated intracellularly to their triphosphate forms before they can compete effectively with the natural deoxynucleotide substrates and act as chain terminators.[3,4] Unfortunately, their use is limited due to rapid emergence of resistant viral strains and to the toxic side effects[5,6] (which may result from the inhibition of cellular DNA polymerases other than RT). On the other hand, the NNRTIs, which belong to a structurally diverse group of compounds, are highly potent and selective against HIV-1 RT and are targeted at sites other than the substrate binding sites of the enzyme. These inhibitors, however, also lead to a rapid accumulation of drug-resistant variants, which is even faster than that observed in the nucleoside analogues. Consequently, the need for the development of effective and selective inhibitors of RT with disparate inhibition mechanisms still remains.

Therefore, we embarked on an in vitro screening program for potent anti-HIV-1 RT drugs from cyanobacteria, a rich but relatively ignored source of novel pharmaceutical "lead" compounds. So far, few anti-HIV compounds from cyanobacteria have been reported. In one case, the described inhibition of RT activity was exhibited by extracts of the cyanobacteria, and, hence, the nature of the inhibitors is unknown.[7] In a second study by Gustafson et al.[8] the anti-HIV compounds, active in a cell-based assay system, were identified as a class of sulfolipids, commonly referred to as sulfoquinovosyl diacylglycerols.

We have recently reported on the capacity of similar known and novel diacylated sulfoglycolipids (isolated from cyanobacteria) to inhibit the DNA polymerase function associated with HIV-1 RT.[9] Herein, we extend our previous work by evaluating and comparing the relative inhibition potency exhibited by the various sulfoglycolipids (compounds **1**−**4**), glycolipids (**5**−**8**), their hydrolysis products (**10**−**11**), and the synthetic derivative, **12**. Attention is primarily focused on the analysis of the structural parameters required for this inhibition.

Results and Discussion

In an attempt to search for natural products with potent anti-HIV RT activity we have found that lipo-

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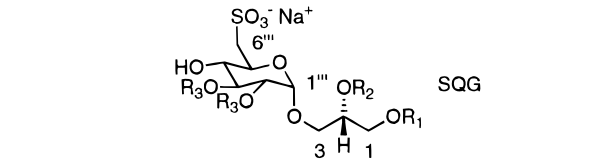
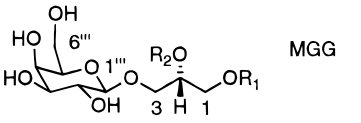
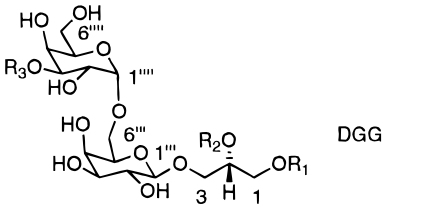
S0163-3864(97)00585-5 CCC: $15.00
Published on Web 06/27/1998



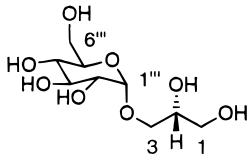
1	R_1 = oleoyl	R_2 = palmitoyl	R_3 = H
2	R_1 = linoleoyl	R_2 = palmitoyl	R_3 = H
3	R_1 = palmitoyl	R_2 = palmitoyl	R_3 = H
4	R_1 = linoleoyl	R_2 = palmitoyl	R_3 = palmitoyl
9	R_1 = H	R_2 = H	R_3 = H



5	R_1 = linoleoyl	R_2 = palmitoyl	
6	R_1 = oleoyl	R_2 = palmitoyl	
8	R_1 = palmitoyl	R_2 = palmitoyl	
10	R_1 = H	R_2 = H	



7	R_1 = linoleoyl	R_2 = palmitoyl	R_3 = palmitoyl
11	R_1 = H	R_2 = H	R_3 = H



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philic extracts of five different cyanobacteria strains [*Oscillatoria raoi* (TAU strain IL-76-1-2), *Scytonema* sp. (TAU strain SL-30-1-4), *Oscillatoria trichoides* (TAU IL-104-3-2), *Phormidium tenue* (TAU IL-144-1), and *Oscillatoria limnetica* Lemmermann (TAU strain NG-4-1-2)] inhibited the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed DNA synthesis of HIV-1 RT by at least 95%, at a concentration of 10 μg/mL of inhibitor. Separation of the active constituents from these extracts has been guided by assaying different fractions for their capacity to inhibit the HIV-1 RT-associated DNA polymerase activity. Thus, sequential chromatography on Si gel and Sephadex LH-20 gel columns followed by reversed-phase HPLC has yielded eight compounds, four sulfoglycolipids (**1**−**4**) and four glycolipids (**5**−**8**), of which two, **4** and **7**, are novel. Other related compounds (**9**−**11**) were derived from compounds **4**, **5**, and **7** by hydrolysis as described.[9] Initial screening for the inhibitory activity of the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed DNA synthesis of HIV-1 RT revealed that compounds **1**−**4** inhibit more than 95% of the initial enzymatic activity, whereas compounds **5**−**8** inhibit the DNA polymerase activity by 65%, 33%, 42%, and 8%, respectively, at a final inhibitor concentration of 10 μM.

Generally speaking, compounds that inhibit in vitro the catalytic activities of HIV-1 RT can be divided into three categories: (a) compounds that block both the DNA polymerase and RNase H activities; (b) inhibitors of the DNA polymerase activity with little or no effect on the RNase H function; and (c) compounds that selectively inhibit the RNase H activity without inhibiting the DNA polymerase activity. The sulfoglycolipids in this study, compounds **1**−**4**, belong to the second category, that is, they are all inhibitors of only the DNA polymerase activity of HIV-1 RT. The 50% inhibitory concentrations (IC_{50}) values for the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed DNA synthesis were determined from the dose-response curve of the inhibitors. Thus, the values calculated for compounds **1**−**4** are 24, 72, 112, and 2950 nM, respectively (Figure 2 and Table 1). The sulfolipids are practically devoid of any significant inhibitory activity against the HIV-1 RT-associated RNase H function. Compounds **1** and **3** display a partial weak inhibitory

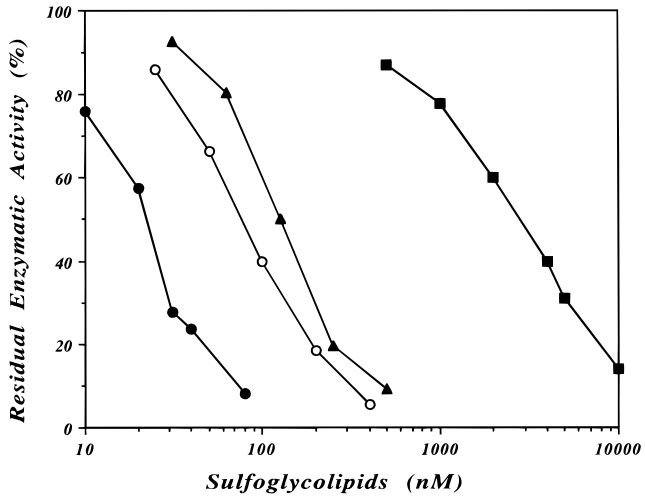

Figure 1. The effect of sulfoglycolipids on HIV-1 RT. The dose−response curves of HIV-1 RT were carried out as described in the Experimental Section. The DNA polymerase activity was assayed by following the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into DNA in the presence of increasing amounts of compound **1** (●), compound **2** (○), compound **3** (▲), compound **4** (■). The residual enzymatic activities were calculated as percentage of the control with no inhibitor.

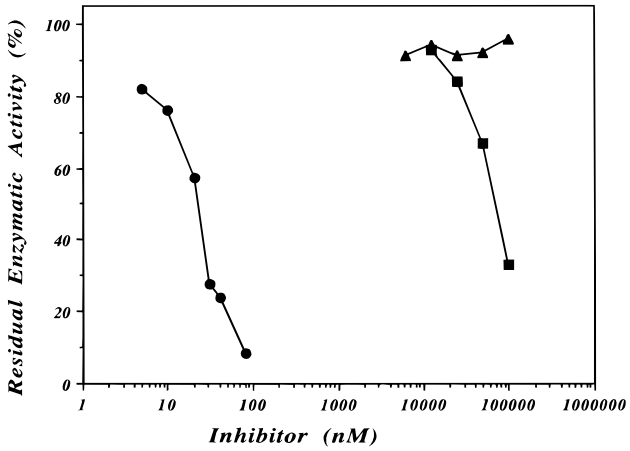

Figure 2. The effect of sulfoglycolipid (**1**) and its derivatives, the sulfoquinovosyl glycerol (**9**) and 1-*O*-(α-D-glucopyranosyl)-glycerol (**12**) on HIV-1 RT. The dose−response experiments were carried out as described in the Experimental Section. The DNA polymerase activity was assayed by following the poly(rA)$_n$·oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into DNA in the presence of increasing amounts of compound **1** (●), compound **9** (■) and compound **12** (▲). The data for compound **1** were replotted from Figure 1.

Table 1. Inhibition of DNA Polymerase and RNase Activities of HIV-1 RT by Sulfoglycolipids, Glycolipids, and Their Derivatives

compound	DNA polymerase[a] IC_{50}	DNA polymerase[a] IC_{90}	RNase H[b] % of initial enzymatic activity
1	0.024 ± 0.003	0.076 ± 0.002	66 ± 7
2	0.077 ± 0.003	0.280 ± 0.80	100
3	0.112 ± 0.023	0.465 ± 0.025	45 ± 4
4	2.95 ± 0.15	14 ± 1.0	100
5	38 ± 4	>100	100
6	65 ± 5.2	>>100	100
7	99.4 ± 16.9	>>100	100
8	>100	>>100	100
9	75 ± 3	>>100	NT[c]
10	>>100	NA[c]	NT
11	>>100	NA	NT
12	>>100	NA	NT

[a] The IC_{50} and IC_{90} values (inhibition concentrations that lead to 50% and 90% inhibition, respectively, of the initial enzymatic activities) are expressed in μM. All data are the mean result of at least two separate experiments. [b] The RNase H activity was assayed in the presence of inhibitor, at a concentration of 100 μM each. The residual enzymatic activity was calculated as percentage of the control with no inhibitor. [c] NA = not applicable; NT = not tested.

capacity at 100 μM by reducing the initial enzymatic activity to 66% and 45%, respectively, whereas compounds **2** and **4** entirely lack such an activity at the same concentration (IC_{50} > 100 μM; Table 1). The novel natural compound **4**, in which the two hydroxyl groups in the sugar moiety are acylated by palmitoyl groups, exhibits a substantial reduction of inhibitory activity compared to compounds **1**−**3** (an increase of about 100-, 40-, and 26-fold in the IC_{50} values, respectively). Apparently, the contribution of two of these palmitoyl moieties to the bulkiness of the molecule at this position interferes with the maximal inhibition activity, possibly by steric hindrance.

As seen in Table 1, the inhibitory effects of compounds **4**−**8**, all of which are natural glycolipids are markedly reduced. For example, compound **6**, the unsulfonated galactosyl version of **1**, substantially loses its capacity to inhibit the HIV-1 RT-associated DNA polymerase activity as manifested by a 2700-fold increase in the IC_{50} value (Table 1). Similar results are obtained by comparing compounds **3** and **8**. Although the glycolipid **8** is completely inactive against the DNA polymerase activity, the corresponding sulfoquinovosyl lipid compound **3** exhibits an IC_{50} value of about 112 nM, a decrease of at least 1000-fold. In general, all glycolipids tested (compounds **5**−**8**) are either poor or inactive inhibitors of the DNA polymerase function and completely incapable of inhibiting the RNase H activity. The results strongly establish the important contribution of the sulfonic acid moiety in the sulfoglycolipid to the potent maximal inhibition capacity of the DNA polymerase function. Likewise, various other sulfonic and sulfate acid derivatives have been shown to exhibit anti-HIV RT activity such as naphthlenesulfonic acid,[10] suramine and its sulfonic derivatives[11,12] toxisuol, toxicol A, and shaagrockols B and C.[13]

To analyze further the structural parameters in the sulfoglycolipids **1**−**3** that are required for potentiating the inhibition of DNA polymerase activity, we have hydrolyzed the fatty acid constituents to produce compound **9**. This sulfoquinovosyl glycerol derivative of compounds **1**−**3** substantially loses the ability to inhibit the DNA polymerase function of RT. Compound **9** displays an IC_{50} value of 75 μM, a 3000- to 700-fold increase in the IC_{50} values observed with the parental sulfoglycolipids **1**−**3** (Figure 2). The acyl chain groups, as in the case of the sulfonic acid moiety, have an influential role in potentiating inhibition of DNA polymerase activity. It seems, however, that the variation within the acyl group (i.e., length and degree of saturation) is not so critical and has a mild effect on the inhibitory activity. Direct evidence that the sulfonic acid moiety is also responsible for the pronounced anti-HIV RT activity was obtained through the chemical synthesis of the unsulfonated derivative of **9**, that is, 1-*O*-(α-D-glucopyranosyl)glycerol (compound **12**). This compound has completely lost the capacity to inhibit the DNA polymerase activity of HIV-1 RT (Table 1). No reduction in the DNA polymerase activity has been detected even in the presence of compound **12** at a concentration as high as 100 μM (Figure 2). Likewise, compounds **10** and **11**, monogalactopyranosyl glycerol and digalctopyranosyl glycerol without a sulfonic acid group constituent, are completely inactive. Here again, hydrolysis of the fatty acid side chain has completely abolished any activity detected in compounds **5**−**8**. A comparison between these derivatives to compound **9** further supports the notion that sulfonic acid residue is essential for enhancing the inhibitory activity of RT. In all, the high potency of the inhibition of RT, exhibited by the sulfoglycolipids, is due to the intact molecule. Both the sulfonic acid and the fatty acid ester side chains are required for maximal effective RT inhibition.

Taken together, it is possible that the lipophilic groups interact with the hydrophobic core of the enzyme, whereas the negatively charged sulfonic moiety may interact with the positively charged side chains on the enzyme. In this regard, the sulfoglycolipids may be similar to the NNRTIs. These RT inhibitors interact with a hydrophobic amino acid pocket of HIV-1 RT that is close to the active site in the DNA polymerase domain.[14,15] Furthermore, in a similar way to the NNRTIs, compounds **1**−**4** preferentially inhibit the DNA polymerase function and not the RNase H activity. In contrast to the NNRTIs that specifically inhibit only HIV-1 RT[2], however, the sulfoglycolipids, of which compound **1** is the most potent inhibitor of HIV-1 RT, are also effective in inhibiting the HIV-2 RT. As seen in Figure 3, compound **1** blocks the HIV-2 RT-associated DNA polymerase potently with an IC_{50} value as low as 34 nM. This may indicate the presence of a hydrophobic pocket in the DNA polymerase domain of HIV-2 RT as well. So far, the three-dimensional structure of only HIV-1 RT was resolved, whereas very little information is known on HIV-2 RT.[14,15] Thus, the similarity in the sensitivity of the two HIV RTs to the sulfoglycolipids studied here may support the data already available on the potential resemblance between the two structures of the two HIV RTs.[16,17]

The anti-HIV-1 activity in T-cell lines displayed by the structural class of sulfolipids[8] could be a reflection of the inhibition of the DNA polymerase activity associated with HIV-1 RT. The high potency of the sulfoglycolipids in the inhibition of HIV-1 RT and in the inhibition of virus replication in cell lines as reported

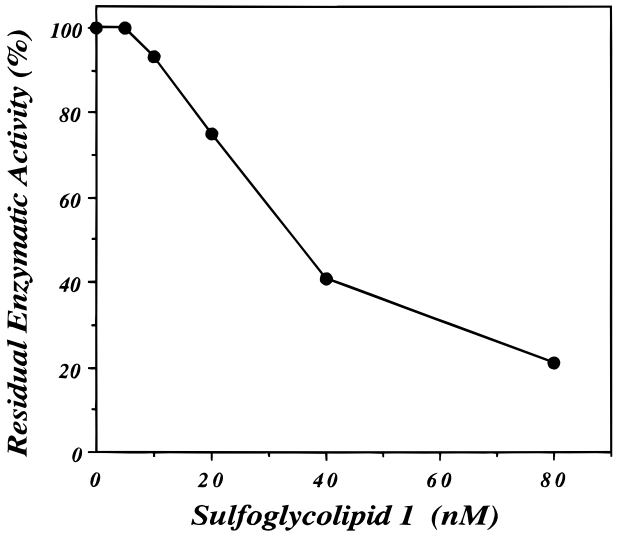

Figure 3. The effect of sulfoglycolipid (**1**) on HIV-2 RT. The dose−response experiments were carried out as described in the Experimental Section and Figure 1.

by others[8] together with the fact that these compounds are abundant (inasmuch as they constitute part of the chloroplast membrane[18]) argues in favor of further development of these compounds as anti-AIDS agents.

Experimental Section

Materials. The sulfoglycolipids **(1−4)** and glycolipids **(5−8)** have been isolated from five cyanobacterial strains *O. raoi* De Toni (TAU strain IL-76-1-2), *Scytonema* sp. (TAU strain IL-104-3-2), *P. tenue* Gomont (TAU strain IL-144-1), *O. limmetica* Lemmermann (TAU strain NG-4-1-2), and *O. trichoides* Sazafer (TAU strain IL-104-3-2) as described in detail.[9]

The isolation and identification of the natural compounds **1−8** were already described in detail.[9] In general, these compounds were purified from the lipophilic crude extracts of the above cyanobacteria by successive steps of gel chromatography: normal phase, then Sephadex LH-20 followed by a final step of reversed-phase HPLC. Enzymatic hydrolysis of compounds **4**, **5**, and **7**, followed by alkaline treatment, afforded the derivatives **9**, **10**, and **11**, respectively, as described.[9] Compound **12** was synthesized according to the procedure described by Yoshida et al.[19]

1-*O*-(2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl)-glycerol (12a). A solution of 2,3,4,6-tetra-*O*-acetyl-D-glucopryanosyl bromide (111 mg, 0.29 mmol) in CH_2Cl_2 (1 mL) was added to suspension of glycerol (13.8 mg. 0.15 mmol), $HgBr_2$ (106 mg. 029 mmol), and MS4 A in CH_2Cl_2 (5 mL). The mixture was stirred overnight at room temperature, filtered, and the filtrate was diluted with CH_2Cl_2. The mixture was successively washed with 10% aqueous KI and brine, dried ($MgSO_4$), and concentrated to a syrup. This was subjected to column chromatography with petroleum ether−EtOAc (2:3) to give **12a** (13 mg, 20%) as an oil: ^{1}H NMR ($CDCl_3$) δ 5.54 (1H, t, J = 9.7 Hz, H-4), 5.46 (1H, d, J = 3.6 Hz, H-1), 5.08 (1H, t, J = 9.6 Hz, H-4), 4.91 (1H, dd, J = 3. 6, 9.7 Hz, H-2), 4.45 (2H, m, H-2′ and H-la′), 4.28 (1H, br d, J = 12.1 Hz, H-6a), 4.25 (1H, m, H−1b′), 4.12 (1H, br d, J = 12.1 Hz, H-6b), 4.10 (2H, m, H-3a′ and H-3b′), 3.57 (1H, br d, J = 9.6 Hz, H-5); positive FABMS (DTT/DTE) m/z 423 $(M + H)^+$, 445 $(M + Na)^+$.

1-*O*-(α-D-glucopyranosyl)glycerol (12). A solution of **12a** (5 mg, 0.012 mmol) in NH_4OH−MeOH (1:10) (2 mL) was stirred at room temperature overnight, then concentrated to dryness under reduced pressure to give compound **12** (3 mg quant.) as white powder: ^{1}H NMR (D_2O) δ 5.20 (1H, d, J = 3.7 Hz, H-1′), 3.88−3.66 (6H, m), 3.54−3.38 (4H, m), 3.21 (1H, t, J = 8.7 Hz); positive FABMS (DTT/DTE) m/z 255 $(M + H)^+$, 277 $(M + Na)^+$.

[^{3}H]dTTP was purchased from ICN and [^{3}H] poly(rA)$_n$ from Amersham. Synthetic primer-template poly(rA)$_n$·oligo(dT)$_{12-18}$, and poly(dT)$_n$ were the products of Pharmacia.

Enzymes. The RTs used in this study were recombinant enzymes expressed in *Escherichia coli* and purified from bacterial extracts to homogeneity as described previously.[20] The HIV-1 RT expression plasmid was derived from the BH-10 proviral isolate,[21] and the expression plasmid of HIV-2 RT was derived from the pRod isolate.[22] The purified RTs of HIV-1 and HIV-2 were p66/p51 and p68/p55 heterodimers, respectively.

Enzymatic assays. The RT-associated DNA polymerase and the RNase H activities were assayed as described in detail previously.[23] In short, the DNA-polymerase activity was assayed by monitoring the poly-(rA)$_n$·oligo(dT)$_{12-18}$-directed incorporation of [^{3}H] dTTP into TCA-insoluble product. The RNase H activity was assayed by measuring the release of TCA-soluble material from the synthetic substrate [^{3}H] poly(rA)$_n$·poly (dT)$_n$. This substrate was prepared according to the procedure described previously[16]. In all inhibition experiments the enzymes were preincubated for 5 min at 30 °C in the absence or presence of inhibitor at various concentrations. The enzymatic reactions were initiated by adding the appropriate substrate followed by an incubation for 30 min at 37 °C. Enzymatic residual activity was calculated relative to the initial linear reaction rates observed when no drug was added. The inhibitor concentration leading to 50% inhibition (IC_{50} values) of the enzymatic activities was calculated from the inhibition curves as a function of the inhibitor concentrations. Enzymatic activities were defined as follows. One unit of DNA polymerase activity is the amount of enzyme that catalyzes the incorporation of 1 pmol of dNTP into DNA product after 30 min at 37 °C under the standard assay conditions. One unit of the RNase H activity is the amount of enzyme that catalyzes the hydrolysis of 1 pmol of AMP after 30 min at 37 °C under the outlined assay conditions.

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NP970585J