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Synthesis of [18,19,21,22-$^{13}C_4$]-Labeled Tautomycin as an NMR Probe of Protein Phosphatase Inhibitor

Minoru Isobe,*[a] Masakuni Kurono,[a] Katsunori Tsuboi,[a] and Akira Takai[b]

Abstract: We have accomplished the synthesis of ^{13}C-labeled tautomycin at the C18, C19, C21, and C22 positions starting from 100% [^{13}C]triethylphosphonoacetate for the purpose of elucidating the dynamics and conformation of the C17–C26 moiety. NMR spectroscopy of ^{13}C-labeled tautomycin revealed strong binding with protein phosphatase type 1 and new features in the ^{13}C NMR spectrum, such as the very small three-bond coupling constants (^2J).

Keywords: enzyme inhibitors · isotope labeling · NMR spectroscopy · protein phosphatase · tautomycin

Introduction

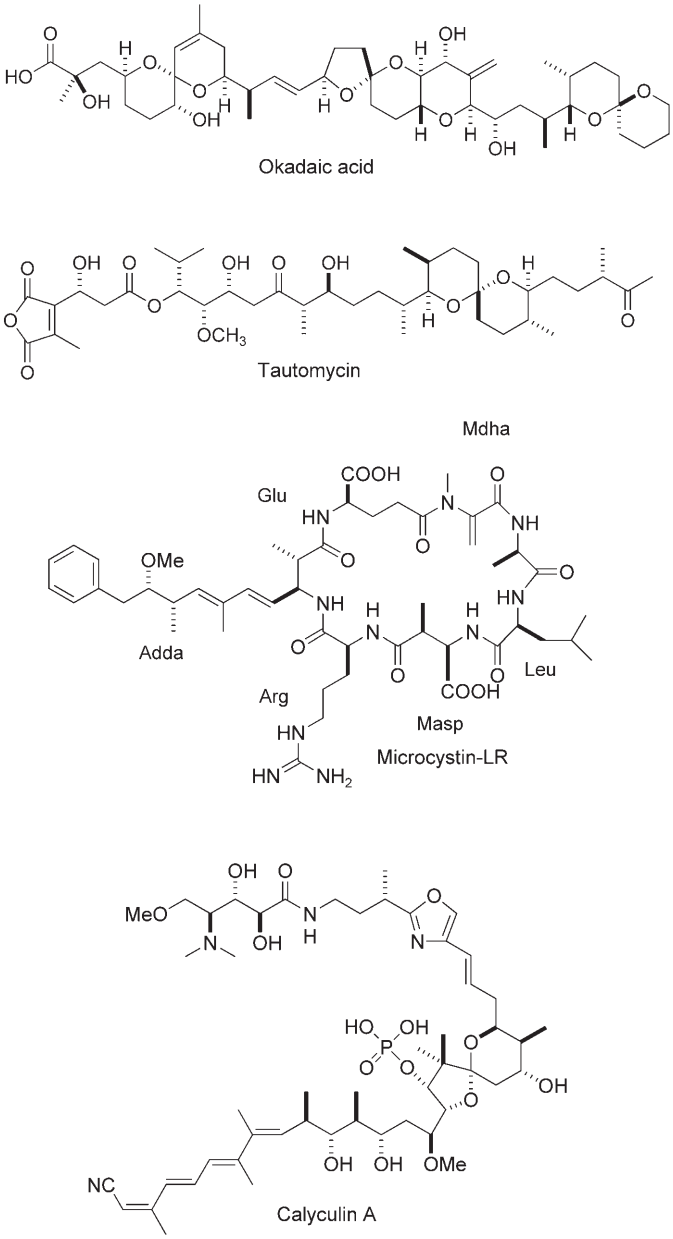
Tautomycin (TTM, **1**) was structurally elucidated by Isono and co-workers,[1–4] and it shows a specific inhibitory activity toward protein phosphatase type 1 (PP1) rather than type 2A (PP2A).[5,6] It has been reported that PP1 or PP2A are inhibited by natural products such as okadaic acid, calyculin, and microcystin-LR (Scheme 1).[7] Each of these three compounds inhibits PP2A much more strongly than PP1, whereas TTM inhibits PP1 more strongly than PP2A.[7] In 1995, Goldberg et al. reported the X-ray crystallographic analysis of PP1–microcystin-LR complex, providing details of molecular interaction between phosphatase and the inhibitor (Figure 1).[8] Subsequently, more X-ray structures were reported of PP1 complexes with okadaic acid (Figure 2) in 2001[9] and with calyculin (Figure 3) in 2002.[10] The crystallographic data suggest that all these inhibitors interact with the five common amino acid residues. The polar functional groups in inhibitors, such as carboxylic or phosphoric acid, interact with Arg96 and Tyr272, respectively. Furthermore, the important hydroxy groups for hydrogen bonding are within a short distance from Arg221 and Tyr134 residues. The three examples imply that inhibitors are bound to the same site on the PP1γ protein. However, the PP1–TTM complex is not yet available, probably owing to the noncrystalline nature of TTM.

[a] Prof. Dr. M. Isobe, Dr. M. Kurono, Dr. K. Tsuboi
Laboratory of Organic Chemistry
Graduate School of Bioagricultural Sciences
Nagoya University
Chikusa, Nagoya 464-8601 (Japan)
Fax: (+81) 52-789-4111
E-mail: isobem@agr.nagoya-u.ac.jp

[b] Prof. Dr. A. Takai
Asahikawa Medical College
Midorigaoka Higashi 2-1-1-1
Asahikawa 078-8510 (Japan)

Tautomycin Diacid is the Active Form of TTM

As TTM itself has no inhibitory activity, the following studies were performed with tautomycin diacid (TTMDA, **1b**), which is, in fact, the active form of this inhibitor. We have reported the structural relationship from the viewpoints of the molecular shape of **1b** in water and in protein solution with special reference to protein phosphatase inhibitory activity.[11–13] Furthermore, we have also reported that one of the stable conformations of **1b**, which was obtained by preliminary computer calculations using Biograf and NMRgraf package programs with NOESY data, is U-shaped in D_2O, and **1b** has a flexible structure from around C20 to C′7.[11] This folded conformation has recently been proven through special fluorescence-quenching experiments of the excited states of the two chromophores at both ends of the TTM molecule.[14] When **1b** is bound to the active site of PP1γ, it is expected that the conformation of **1b** might be transformed to the active one. A possible conformer would be that shown in Figure 1, in which the white frame is an expected conformation of TTMDA. This model was generated so that each important functional group can have maximum interaction with one of the amino acid residues Arg96, Tyr134, Arg221, and Tyr272. We assumed that the conformation of the C21–C26 moiety of **1b** might bear one of the key issues related to inhibitory activity. Herein, we repeat the synthesis of 100% enriched ^{13}C-labeled TTMDA at the C18,



Scheme 1. Four typical natural protein phosphatase inhibitors.

Abstract in Japanese:

タンパク質脱リン酸酵素 PP1γ特異的阻害剤として知られるトートマイシンは、単独では屈曲型立体配座をとることを複数の手法で証明してきた。この阻害剤がタンパク質中で阻害作用を示す時の結合型の分子形状を調べる目的で、^{13}C NMR による標識化合物を合成した。すなわちトートマイシンの 18, 19, 21, 22-位に 100% ^{13}C とした分子種を合成した。実際 PP1γときわめて強く結合し、NMR シグナルはブロードになり、天然含量（1% ^{13}C）トートマイシンでは、シグナルが復活した。

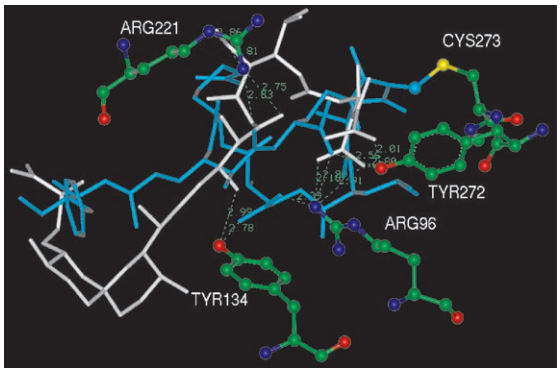


Figure 1. Mycrocystin-LR (blue) with interacting amino acid residues of PP1γ. The white frame shows a possible conformation of TTMDA.

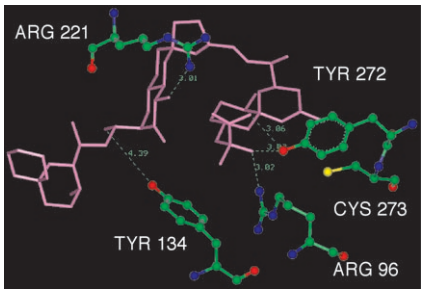


Figure 2. Okadaic acid and PP1γ interacting with the same amino acid residues.

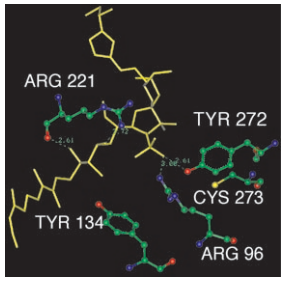
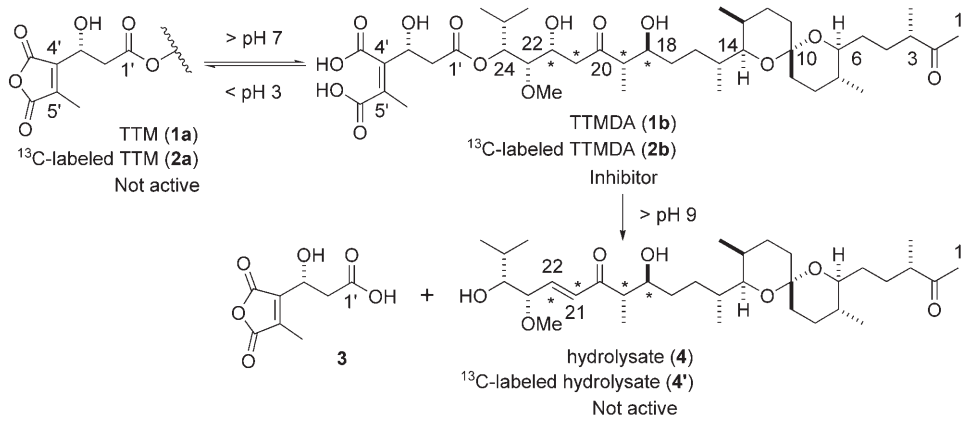


Figure 3. Calyculin and PP1γ interacting with Arg96, Arg221, and Tyr272 by using the phosphoric acid moiety.

C19, C21, and C22 positions to elucidate the dynamics and conformation of the C17–C26 moiety in the PP1γ–**2b** complex by measuring the dihedral angles through the ^{13}C–^{13}C and ^{13}C–^{1}H coupling constants. Natural TTM is known to be unstable and affords **3** and **4** during storage of **1** via *trans* esterification followed by β elimination (Scheme 2). As unsaturated ketone **4** shows no inhibitory activity, it was used as an internal standard for the NMR binding experiments.

We have already reported the total synthesis of tautomycin in 1995[15] and an improved total synthesis in 1997.[16] The sulfone carbanion (segment C, **7**) was added to the epoxide segment (segment B, unlabeled **6**), which was then esterified with the carboxylic segment (segment A, **5**). For the introduction of 100% ^{13}C-enriched carbon atoms at the C18, C19, C21, and C22 positions, the synthetic route was slightly modified from the previous total synthesis. We established a



Scheme 2. Tautomycin (TTM) and tautomycin diacid (TTMDA). The asterisk (*) indicates ^{13}C atoms.

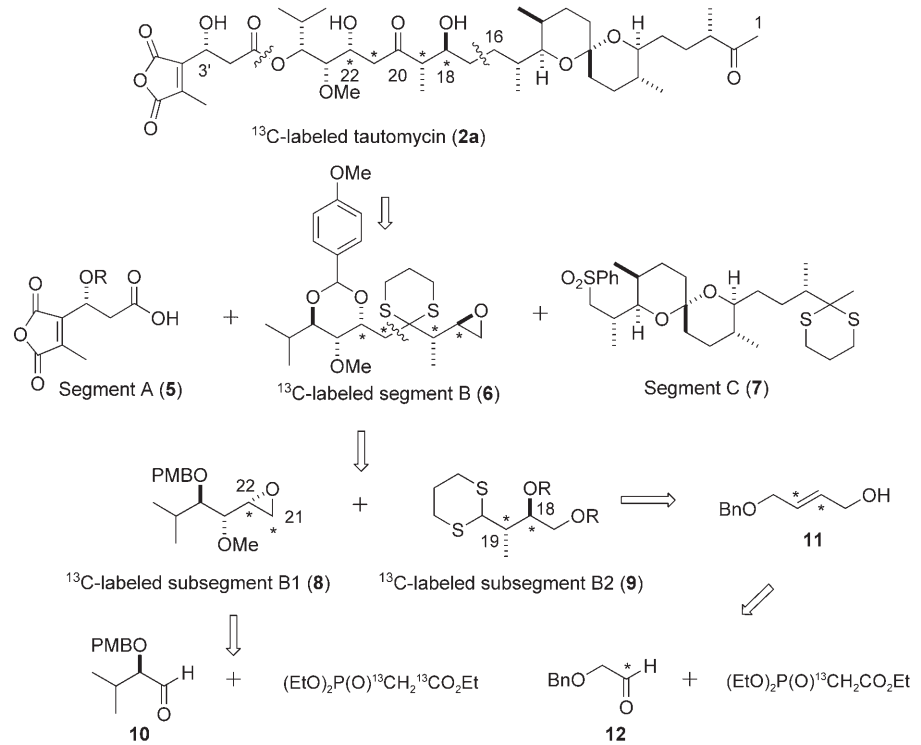
method to introduce the isotopic carbon atoms at these positions as well as any other part between C17 and C21 by starting from commercially available [1-^{13}C], [2-^{13}C], and/or [1,2-^{13}C]-trimethylphosphonoacetate. Scheme 3 illustrates the retrosynthetic analysis of the [18,19,21,22-$^{13}C_4$]-labeling route of tautomycin (**2a**). Retrosynthetic disconnection of [18,19,21,22-$^{13}C_4$]-labeled segment B (**6**) provides [21,22-$^{13}C_4$]-labeled subsegment B1 (**8**) and [18,19-$^{13}C_4$]-labeled subsegment B2 (**9**) which could be derived from **11**. Epoxide **8** could be prepared from the olefination of aldehyde **10** and [1,2-$^{13}C_2$]-labeled trimethylphosphonoacetate by using the same strategy as in our previous work for the synthesis of segment B.[17–18] On the other hand, disconnection of **11** provides the ^{13}C-labeled aldehyde **12** and [2-^{13}C]-labeled trimethylphosphonoacetate.

Synthesis of [21,22-$^{13}C_2$]-Labeled Subsegment B1

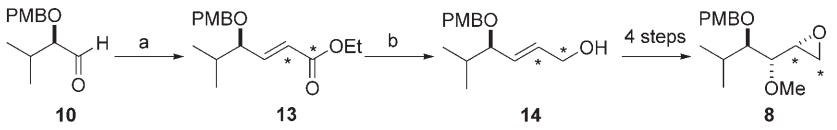
The synthesis of [21,22-$^{13}C_2$]-labeled subsegment B1 (**8**) commenced from the previously reported aldehyde **10** (Scheme 4), which was prepared from D-valine.[19,20] Horner–Wadsworth–Emmons olefination with [1,2-$^{13}C_2$]-labeled trimethylphosphonoacetate and NaH gave the $^{13}C_2$-labeled unsaturated ester **13**, which was successively treated with diisobutylaluminum hydride (DIBAL) to provide $^{13}C_2$-labeled allylic alcohol **14**. Compound **8** was prepared from **14** in 53% overall yield in accordance with the previous synthesis of subsegment B1 under similar conditions.[17]

Synthesis of [18,19-$^{13}C_2$]-Labeled Subsegment B2

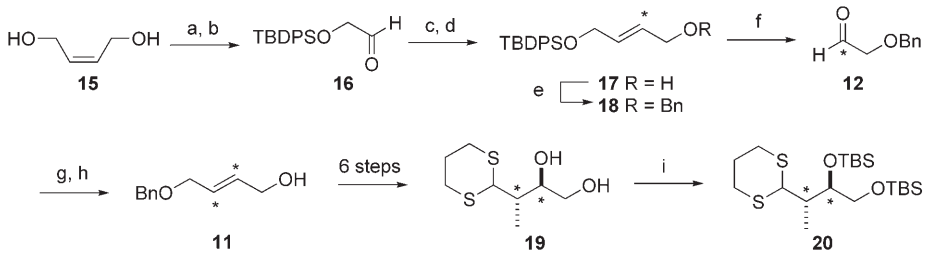
Commercially available *cis*-butane-1,4-diol (**15**) was first protected, then its double bond was cleaved by ozonolysis to give *tert*-butyldiphenylsilyloxyacetaldehyde (**16**) (Scheme 5).[21] Horner–Wadsworth–Emmons olefination with [2-^{13}C]-labeled trimethylphosphonoacetate and NaH was followed by reduction of the resulting unsaturated ester by DIBAL to give the ^{13}C-labeled allylic alcohol **17**. Protection of the hydroxy group in **17** by



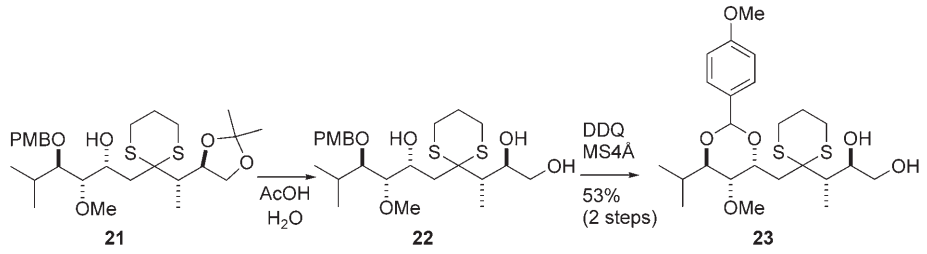
Scheme 3. Retrosynthetic analysis of ^{13}C-labeled tautomycin. PMB = *p*-methoxybenzyl.



Scheme 4. Synthesis of [21,22-$^{13}C_2$]-labeled subsegment B1. Reagents and conditions: a) $(EtO)_2P(O)^{13}CH_2{}^{13}CO_2Et$, NaH, THF, −78 °C, 30 min; b) DIBAL, CH_2Cl_2, −78 °C, 20 min, then 0 °C, 1 h, 95% (2 steps).



Scheme 5. Synthesis of [18,19-$^{13}C_4$]-labeled subsegment B2. Reagents and conditions: a) TBDPSCl, imidazole, DMF, room temperature, 12 h, 96%; b) O_3, CH_2Cl_2/MeOH (7:3), −78°C, then, Ph_3P, room temperature, 2 h, 97%; c) $(EtO)_2P(O)^{13}CH_2CO_2Et$, NaH, THF, −78°C, 15 min; d) DIBAL, CH_2Cl_2, −78°C, 20 min, then 0°C, 1 h, 98% (2 steps); e) BnBr, KH, THF, 0°C, 20 min, 86%; f) O_3, CH_2Cl_2/MeOH = (7:3), −78°C, then, Ph_3P, room temperature, 1 h, 98%; g) $(EtO)_2P(O)^{13}CH_2CO_2Et$, NaH, THF, −78°C, 20 min; h) DIBAL, CH_2Cl_2, −78°C, 20 min, then 0°C, 30 min, 84% (2 steps); i) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0°C, 1 h, 87%. DMF = *N*,*N*-dimethylformamide, TBDPS = *tert*-butyldiphenylsilyl, TBS = *tert*-butyldimethylsilyl, Tf = trifluoromethanesulfonyl.



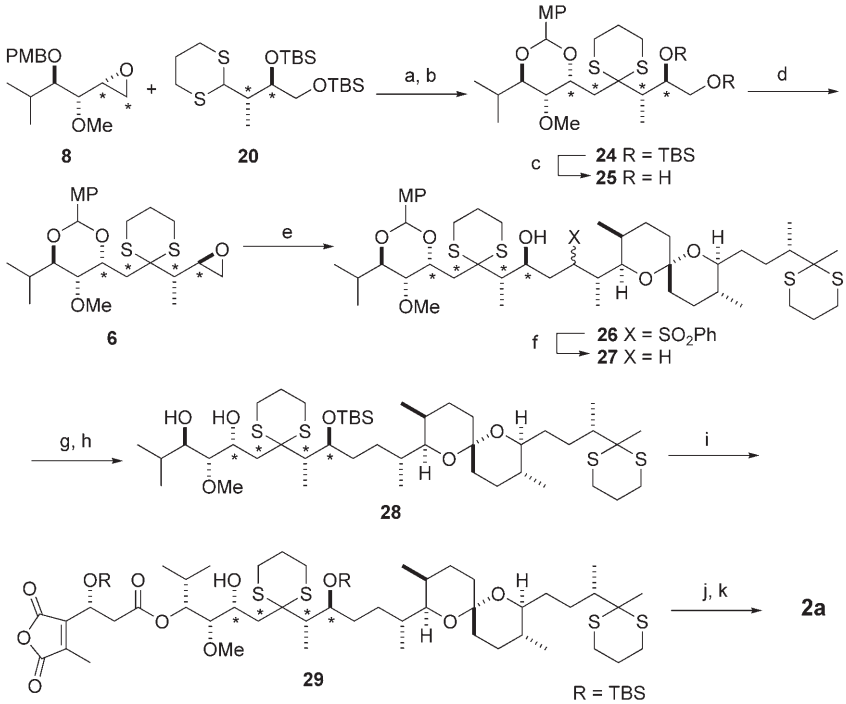
Scheme 6. Previous synthetic route to **23**.[18] MS4Å = 4-Å molecular sieves.

treatment with BnBr in the presence of KH afforded the corresponding benzyl ether **18**, which was subjected to ozonolysis to provide the ^{13}C-labeled oxyacetaldehyde **12**. Once again, Horner–Wadsworth–Emmons olefination was utilized for the double labeling of **12**, which was then followed by reduction with DIBAL to afford the $^{13}C_2$-labeled allylic alcohol **11**. Compound **11** was transformed into dithianes **19** and **20**, which is an intermediate to subsegment B2, according to our previously established route.[17]

In our previous route (Scheme 6), there was some problems after the hydrolysis of the acetonide in **21** with aqueous acetic acid to provide the triol **22**. The treatment of **22** with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) for intramolecular benzylidene formation produced an unknown by-product. This reaction took place at the primary alcohol. Therefore, these two hydroxy groups at the C17 and C18 positions in **19** were protected as the *tert*-butyldimethylsilyl (TBS) ether to furnish [18,19-$^{13}C_4$]-labeled subsegment B2 (**20**) from **11** in 44% overall yield.

Synthesis of [18,19,21,22-$^{13}C_4$]-Labeled Tautomycin

Scheme 7 summarizes the final steps of the synthesis of [18,19,21,22-$^{13}C_4$]-labeled tautomycin (**2a**). Treatment of **20** with *t*BuLi at −55°C for 1 h in 10% hexamethylphosphoramide (HMPA)/THF gave the corresponding dithiane carbanion, to which was added **8** at −55°C to furnish the coupling product. Treatment of this product with DDQ gave the $^{13}C_4$-labeled benzylidene derivative **24** in 75% overall yield.



Scheme 7. Reagents and conditions: a) **20**, *t*BuLi, THF, HMPA, −55°C, 1 h, then **6**, 1.5 h; b) DDQ, MS4 Å, CH_2Cl_2, room temperature, 75% (2 steps); c) TBAF, MeCN, 60°C, 4 h, 91%; d) *n*BuLi, THF, 0°C, 15 min, then TsCl, 20 min, 74%; e) **7**, *n*BuLi, THF, −78°C, 30 min, $BF_3{\cdot}OEt_2$, 30 min, then **6**, −78°C to −50°C, 1 h, 65%; f) Na/Hg, MeOH, room temperature, 12 h, 73%; g) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0°C, 10 min; h) TS, MeOH, room temperature, 12 h, 82% (2 steps); i) **5**, $Cl_3C_6H_2COCl_2$, Et_3N, DMAP, toluene, room temperature, 2 h, 77%; j) $(HF)_x{\cdot}Py$, THF, room temperature, 12 h; k) $Hg(ClO_4)_2$, $CaCO_3$, MeCN, H_2O, room temperature 10 min, 78% (2 steps). DMAP = 4-dimethylaminopyridine, MP = *p*-methoxyphenyl, PPTS = pyridinium *p*-toluenesulfonate, Py = pyridine.

Hydrolysis of the TBS groups in **24** afforded **25** in 91% yield by treatment with tetrabutylammonium fluoride (TBAF). Diol **25** was further transformed into **6** in 74% yield by employing a similar method to the one described previously.[17] Subsequently, the synthesis of **2a** was achieved through the reported pathway,[15] namely, the coupling between **6** and **7**[22,23] followed by desulfonylation of the resulting **26** with sodium amalgam (Na/Hg) to afford diol **27**. The benzylidene protecting group in **27** was removed to give **28** in two steps, and the final selective esterification was performed with **5**[24,25] under Yamaguchi conditions.[26] A two-step deprotection sequence via ester **29** removed the two dithioketal groups and two TBS groups[27] to give **2a** in 23% overall yield from **6**.

NMR Experiments of PP1γ/Inhibitor Complex

Figure 4 shows the ^{13}C NMR spectrum of **2a**. The proton-decoupled spectrum shows the signal of C18 (d, $J=37.3$ Hz) and C22 (d, $J=39.9$ Hz) as simple doublets. Two sets of doublet of doublet (dd) were assigned to C19 (dd, $J=37.3$, 10.3 Hz) and C21 (dd, $J=39.9$, 10.3 Hz). These data show that the ^1J and ^2J couplings are large enough, but the ^3J (three-bond coupling) is very small in this case. Even when the dihedral angle was 180°, ^3J values were found to be 4 Hz or smaller depending on the substituents on the intervening carbon atoms. The details of these phenomena will be published elsewhere in due course.

As mentioned in Scheme 2, TTM **2a** was hydrolyzed to the diacid **2b** and [18,19,21,22-$^{13}C_4$]-labeled hydrolysate **4′**, and a solution of **4′** in 3% [D_6]DMSO/D_2O was placed in an NMR tube. Hydrolysate **4′** is suitable as an internal marker,[3] because it does not show any inhibitory activity. The signals of the C18, C19, C21, and C22 positions of **2b** were observed at 75.4, 66.9, 54.4, and 48.7 ppm, respectively. The $^1J_{C18,C19}$, $^1J_{C21,C22}$, and $^2J_{C19,C21}$ values were 37.2, 40.7, and 9.5 Hz, respectively. On the other hand, the signals of C18, C19, C21, and C22 of **4′** were observed at 145.0, 134.0, 75.2, and 51.8 ppm, respectively. Their $^1J_{C18,C19}$, $^1J_{C21,C22}$, and $^2J_{C19,C21}$ values were 36.7, 69.2, and 11.3 Hz, respectively. The NMR spectrum is shown in Figure 5a.

The PP1γ protein sample used for the NMR experiments was prepared by overexpression in *Escherichia coli*[28] and purified in three steps by using a Q-sepharose column, a heparin sepharose column, and a sephacryl column in sequence. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of this purified PP1γ sample showed a single band at an apparent molecular mass of 37 kDa. The

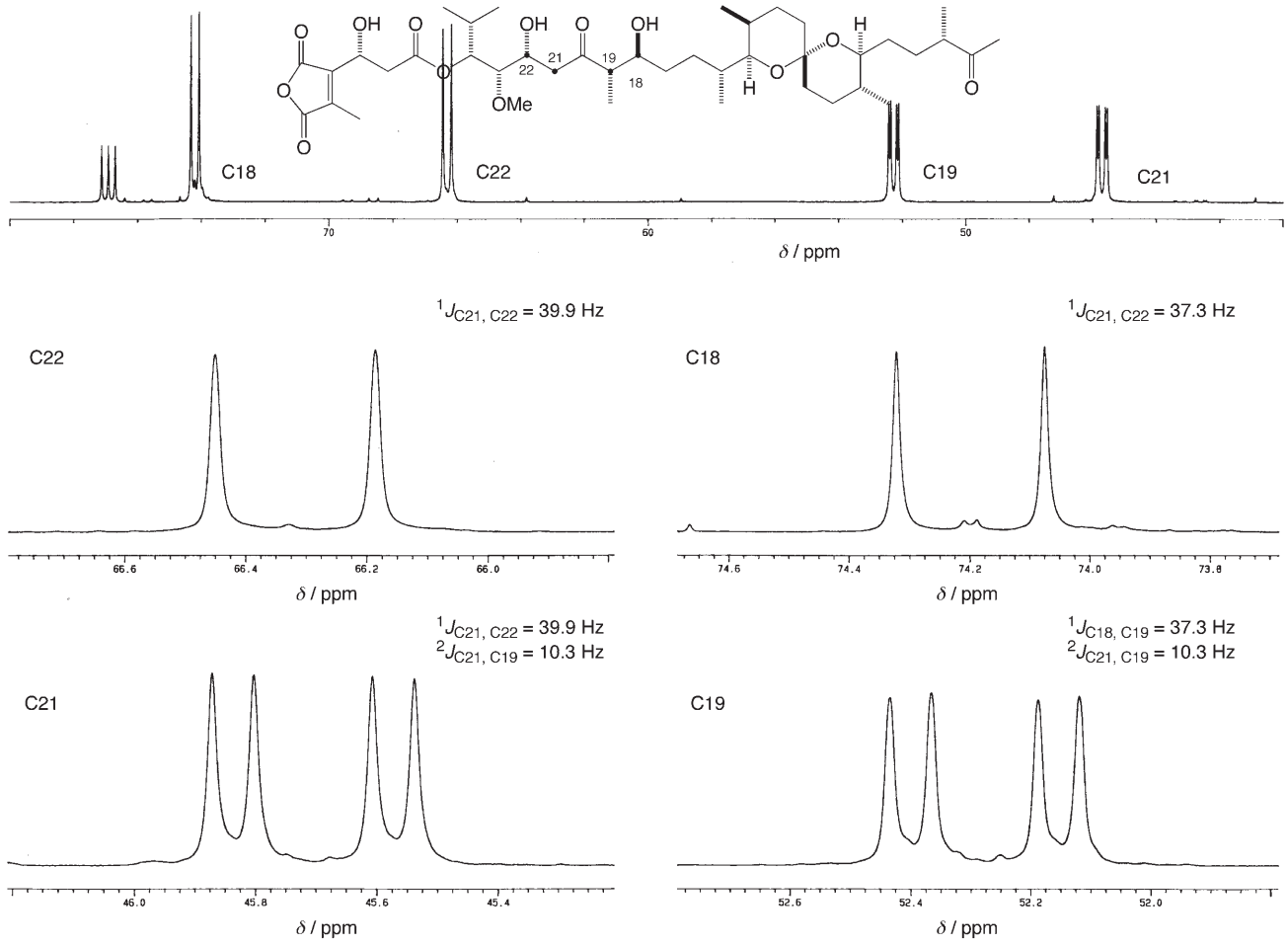


Figure 4. ^{13}C NMR spectrum of 100% ^{13}C-enriched tautomycin at the C18, C19, C21, and C22 positions.

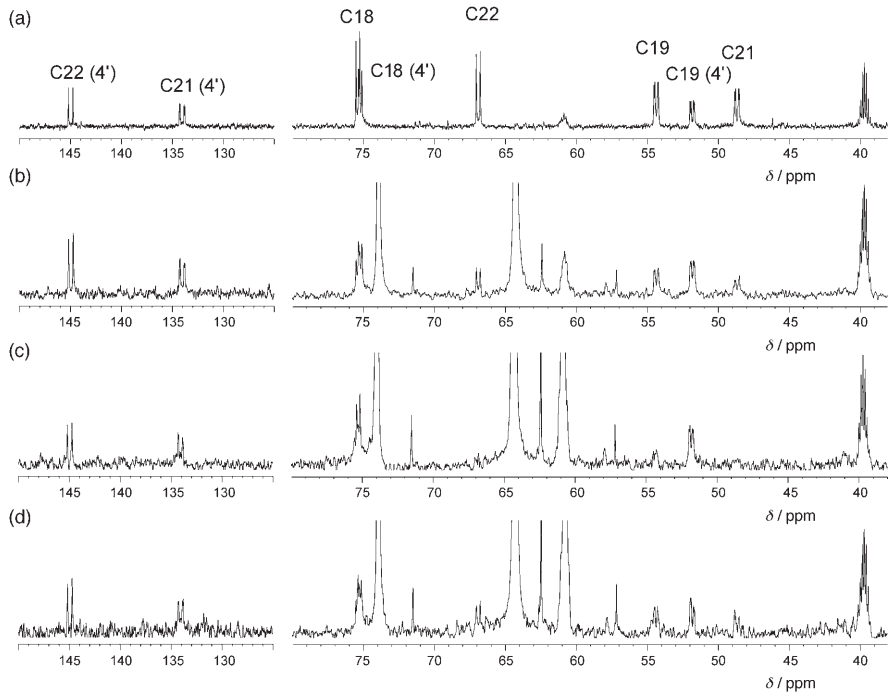


Figure 5. ^{13}C NMR spectrum of 100% ^{13}C-enriched TTMDA at the C18, C19, C21, and C22 positions. a) Spectrum acquired in D_2O (small signals are due to partial decomposition to the corresponding enone **4′**, which has no interacting effect in this study; it was used as an internal marker). b) Spectrum acquired with 0.5 equivalents of PP1γ. c) Spectrum acquired with 0.75 equivalents of PP1γ/inhibitor complex. d) Spectrum acquired after addition of 1 equivalent of unlabeled TTMDA to the previous solution.

^{13}C NMR spectra of **2b** with and without PP1γ were almost identical. The specific activity of this PP1γ sample was determined by the titration procedure described by Takai and Mieskes[30] as 0.15 only. We assumed that such a low specific activity might be due to damage during the handling process. For our semistoichiometric experiments between the protein and the labeled inhibitor molecule, the specific activity is very significant. To enhance the specific activity of PP1γ, we purified it by using a different method, thus furnishing PP1γ with a high specific activity in the region of 0.85 (see Experimental Section).

The ^{13}C NMR spectrum of **2b** is shown in Figure 5a, in which partial decomposition of the sample can be seen. The spectrum of the PP1γ/**2b** (0.5:1) complex, prepared by addition of half an equivalent of PP1γ, is shown in Figure 5b. The signals of **2b** here are smaller than in Figure 5a. When more PP1γ was added so that the mole ratio of PP1γ/**2b** was 0.75:1, the signals of **2b** almost disappeared (Figure 5c). When an equal amount of unlabeled TTMDA (**1b**) was added to the solution of PP1γ/**2b** (0.75:1) so that the mole ratio of PP1γ/**2b**/**1b** was 0.75:1:1, the signals of **2b** reappeared with intensities of about half of what was seen for the original **2b** (Figure 5d). These results show that PP1γ forms a stoichiometric complex with **2b**. However, new signals for the bound state of **2b** were not observed under these conditions. The disappearance of the signals of **2b** is presumably due to the line broadening that occurred upon strong binding to PP1γ ($K_i = 0.4$ nM) and increasing the apparent molecular weight.[7] Unlabeled tautomycin diacid **1b** competed with **2b** for the binding site on PP1γ. This result clearly shows that the decreased signals of **2b** are due to binding to PP1γ.

Conclusions

We have achieved the synthesis of [18, 19, 21, 22-$^{13}C_4$]-labeled tautomycin (**2a**). This synthetic route can introduce isotopic carbon atoms onto the C18, C19, C21, and C22 positions as well as any other part from C17 to C21. NMR analysis of the PP1γ/inhibitor complex helped us to improve the purification method of PP1γ; however, the signals of the bound form of ^{13}C-labeled TTMDA (**2b**) could not be observed due to the line broadening that occurred upon strong binding to PP1γ. NMR studies with TTMDA in aqueous solution also provided us with extensive conformational information due to the natural abundance of ^{13}C and 100% enriched ^{13}C coupling. Recently, we performed photoaffinity-labeling experiments to study the molecular interaction between tautomycin and protein phosphatase proteins. Futher studies are in progress.

Experimental Section

General

^1H and ^{13}C NMR spectra were recorded on a Varian Gemini-2000, a BRUKER ARX-400, and/or an AMX-600 spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane ($\delta = 0.00$ ppm) for ^1H and $CDCl_3$ ($\delta = 77.0$ ppm) for ^{13}C. Data are reported as follows: chemical shift, multiplicity (d = doublet), coupling constant(s), assignment. The numbering for tautomycin shown in Scheme 3 is employed for the assignment of ^{13}C NMR spectra. Mass spectra were recorded either on a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray-type ESI source or on a JEOL LCmate spectrometer. A high-resolution mass spectrum (HRMS) was recorded on a JEOL LCmate spectrometer and is reported in *m/z*. Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25-mm silica-gel-coated glass plates $60F_{254}$ with UV light as visualizing agent and heated with molybdo(VI)phosphoric acid *n*-hydrate or *p*-anisaldehyde solution as developing agents. Silica gel 60 (particle size 0.063–0.2 mm ASTM) was used for open-column chromatography. Dry THF was distilled from potassium metal with benzophenone indicator under N_2 atmosphere. Dry CH_2Cl_2 was distilled from CaH_2 under nitrogen atmosphere. Pyridine and Et_3N were dried over anhydrous KOH pellets. All other commercially available reagents were used as received. All organic solutions from workup

were dried by brief exposure to anhydrous sodium sulfate. Tautomycin (**1**) was kindly provided by Dr. K. Isono (formerly of the Riken Institute) and further purified by the method described previously with slight modification.[29–31]

Apparatus

Incubation was performed with an Incuvator (Sanyo), a Vortex Shaker VR-36 (Taitec), and an Orbital Incuvator MIR-220R Gallenkamp (Sanyo) incubator. Centrifugation was performed with a Model 7930 (Kubota), an RA-3000 (Kubota), and an Eppendorf 5415 centrifuge. Sonication was performed with an Ultra S Homogenizer VP-5S (Taitec) instrument. The purification system consisted of a Pharmacia LKB P-1 peristaltic pump, a Pharmacia P-50 HiLoad pump, a Pharmacia LKB HPLC 2248 pump, a Pharmacia LKB Uvicord SD, a Pharmacia LKB GradiFrac, and a Servocoreder SR 6221 (Graphtec) recorder. Absorbance for a brief *p*-nitrophenyl phospate (PNPP) phosphatase activity assay was determined with a Microskan Ms 96-well microplate reader (Labsystems). SDS-PAGE was performed with Pharmacia LKB Biotechnology PhastSystem. Dialysis was performed with Visking seamless cellulose tubing (15.9 or 6.4 mm internal diameter). Filtration of enzyme solution was performed with Advantec membrane filters (cellulose acetate 5.0 mm pore, 47 mm internal diameter, or 1.0 mm pore, 47 mm internal diameter, and/or cellulose nitrate 0.45 mm pore, 47 mm internal diameter). Ultrafiltration was performed by using an Amicon ultrafiltration cell Model 8050 with Amicon Diaflo ultrafiltration membranes YM 10 (10 per pack, 43 mm internal diameter). 2D electrophoresis was performed with an IPGphor (Pharmacia), a Multiphor II (Pharmacia), an Electrophoresis Power Supply-EPS 3501 XL (Pharmacia), and a MultiTemp II instrument.

Syntheses

Experimental details for the synthesis of ^{13}C-labeled tautomycin are only reported for new compounds, as the intermediates were synthesized by using reported pathways and the original work is referenced.[15,17,22,24] ^{13}C NMR spectroscopy and MS data are only reported for selected intermediates. NMR data for the ^{13}C-labeled compounds are only reported for the ^{13}C isotope due to complications arising from C–H and C–C coupling in ^1H and ^{13}C NMR spectra.

14: [$^{13}C_2$]triethylphosphonoacetate (0.36 mL, 1.8 mmol) was added to a suspension of NaH (60% dispersion in mineral oil, 75 mg, 1.9 mmol, washed with hexane before use) in THF (8 mL) under N_2 atmosphere at 0 °C over 5 min. After the mixture was stirred at 0 °C for 10 min, the resulting homogeneous solution was cooled to −78 °C. A solution of **10** (0.38 g, 1.7 mmol) dissolved in THF (4.5 mL) was added, and the reaction mixture was stirred at −78 °C for 30 min. The cooling bath was removed, and stirring was continued at room temperature for 30 min. Water was added to the mixture, and the resulting aqueous phase was extracted with Et_2O (3×25 mL). The combined organic phase was washed with water (3×) and brine, dried, and then concentrated to give crude **13**, which was dissolved in CH_2Cl_2 (20 mL). DIBAL (0.95 M solution in hexane; 4.5 mL, 4.3 mmol) was added to this solution at −78 °C, and stirring was continued for 20 min. The reaction mixture was warmed to 0 °C over 1 h, and MeOH was added to the mixture. The reaction mixture was poured into ice-cooled aqueous potassium sodium tartrate (10 g per 100 mL). The separated aqueous phase was extracted with CH_2Cl_2 (3×25 mL), and the combined organic extract was dried and then concentrated under reduced pressure to give the residue, which was purified by silica-gel chromatography (30 g, ether/hexane = 1:1) to afford **14** (0.41 g, 95%, 2 steps). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 63.1 (d, J = 47 Hz, C21), 132.8 ppm (d, J = 47 Hz, C22); MS (ESI Q-TOF): m/z calcd for $C_{13}{}^{13}C_2H_{22}NaO_3$: 275.1534 $[M+Na]^+$; found: 275.1606.

8: ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 43.8 (d, J = 30 Hz), 53.0 ppm (d, J = 30 Hz); MS (ESI Q-TOF): m/z calcd for $C_{14}{}^{13}C_2H_{24}NaO_4$: 305.1639 $[M+Na]^+$; found: 305.1641.

17: [$^{13}C_2$]triethylphosphonoacetate (0.60 mL, 3.0 mmol) was added to a suspension of NaH (60% dispersion in mineral oil, 0.13 g, 3.2 mmol, washed with hexane before use) in THF (20 mL) under N_2 atmosphere at 0 °C over 5 min. After the mixture was stirred at room temperature for 30 min, the resulting homogeneous solution was cooled to −78 °C. A solution of **16** (0.86 g, 2.9 mmol) dissolved in THF (10 mL) was added, and the reaction mixture was stirred at −78 °C for 15 min. The cooling bath was removed, and stirring was continued at room temperature for 30 min. Water was added to the mixture and extracted with ether (3× 25 mL). The combined organic phase was washed with water (3×) and brine, dried, and then concentrated. The residue thus obtained was dissolved in CH_2Cl_2 (30 mL). DIBAL (0.95 M solution in hexane; 6.7 mL, 6.4 mmol) was added to this solution at −78 °C, and stirring was continued for 20 min. The mixture was warmed to 0 °C over 1 h, then the reaction was quenched with MeOH. The reaction mixture was poured into ice-cooled aqueous potassium sodium tartrate (10 g per 100 mL). The separated aqueous phase was extracted with CH_2Cl_2 (3×25 mL), and the combined organic layers were dried and then concentrated. Purification by silica-gel chromatography (50 g, ether/hexane = 1:1) gave **17** (0.92 g, 98%, 2 steps). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 128.9 ppm (C19); MS (ESI Q-TOF): m/z calcd for $C_{19}{}^{13}CH_{26}NaO_2Si$: 350.1633 $[M+Na]^+$; found: 350.1693.

18: A solution of **17** (1.0 g, 3.1 mmol) in THF (15 mL) was added to a suspension of KH (35% dispersion in mineral oil, 0.42 g, 3.7 mmol, washed with hexane before use) in THF (15 mL) at 0 °C. After 30 min, benzyl bromide (0.44 mL, 3.7 mmol) was added. The solution was stirred for 20 min, after which the reaction was quenched with ice and the mixture extracted with ether. The combined organic layer was dried and then concentrated under reduced pressure to give the residue, which was purified by silica-gel chromatography (50 g, ether/hexane = 1:20 then 1:10) to afford **18** (1.02 g, 79%). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 126.2 ppm (C19).

12: Aldehyde **18** (1.11 g, 2.66 mmol) was dissolved in CH_2Cl_2 (21 mL) and MeOH (9 mL), and the solution was cooled to −78 °C. Ozone was bubbled through the reaction mixture until the solution turned blue. Triphenylphospine (1.39 g, 5.32 mmol) was added, and the mixture was stirred at room temperature for 1 h. The solvent was evaporated, and the resulting residue was purified by silica-gel chromatography (50 g, ether/hexane = 1:15, 1:4, then 3:1) to give *tert*-butyldiphenylsiloxy acetaldehyde (0.71 g, 90%) and **12** (0.40 g, 98%). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 200.6 ppm (C19).

11: [$^{13}C_2$]triethylphosphonoacetate (0.41 mL, 2.1 mmol) was added to a suspension of NaH (60% dispersion in mineral oil, 87 mg, 2.2 mmol, washed with hexane before use) in THF (14 mL) under N_2 atmosphere at 0 °C over 5 min. After the mixture was stirred at room temperature for 30 min, the resulting homogeneous solution was cooled to −78 °C. A solution of **12** (0.30 g, 2.0 mmol) dissolved in THF (7 mL) was added, and the reaction mixture was stirred at −78 °C for 20 min. The cooling bath was removed, and stirring was continued at 0 °C for 20 min. The reaction mixture was quenched with water and extracted with Et_2O (3×25 mL). The combined organic phase was washed with water (3×) and brine, dried, and then concentrated. The resulting residue was purified by silica-gel chromatography (20 g, ether/hexane = 1:2 then 3:1) to afford the ester (0.34 g, 77%) and recovered **12** (45 mg, 15%). The ester (0.82 g, 3.7 mmol) was then dissolved in CH_2Cl_2 (16 mL) and treated with DIBAL (0.95 M solution in hexane; 8.6 mL, 8.2 mmol) at −78 °C, then stirring was continued for 20 min. The reaction mixture was warmed to 0 °C for 30 min, and MeOH was added to the mixture. The reaction mixture was poured into ice-cooled aqueous potassium sodium tartrate (8 g per 80 mL). The separated aqueous phase was extracted with CH_2Cl_2 (3× 20 mL), and the combined organic extracts were dried and then concentrated under reduced pressure followed by purification by silica-gel chromatography (30 g, ether/hexane = 2:1) to afford **11** (0.61 g, 92%). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 127.9 (d, J = 73 Hz), 132.2 ppm (d, J = 73 Hz); MS (FAB) (glycerol): m/z = 181 $[M+H]^+$.

20: *tert*-Butyldimethylsilyl trifluoromethanesulfonate (0.55 mL, 2.38 mmol) was added to a solution of **19** (200 mg, 0.95 mmol) and 2,6-lutidine (0.43 mL, 3.80 mmol) in CH_2Cl_2 (12 mL) at 0 °C. After being stirred for 1 h, the mixture was poured into aqueous saturated $NaHCO_3$, and the aqueous layer was extracted with Et_2O (3×10 mL). The combined organic layers were washed with water (3×) and brine, dried, and concentrated. Purification by silica-gel chromatography (30 g, hexane then

ether/hexane = 1:25) afforded **20** (362 mg, 87%). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ = 42.1 (d, J = 39 Hz, C19), 73.7 ppm (d, J = 39 Hz, C18); MS (ESI Q-TOF): m/z calcd for $C_{18}{}^{13}C_2H_{44}NaO_2S_2Si$: 461.2286 $[M+Na]^+$; found: 461.2272.

24: *t*BuLi (1.54 M solution in pentane, 0.39 mL, 0.60 mmol) was added to a solution of **20** (200 mg, 0.46 mmol) in a mixture of THF (2.3 mL) and HMPA (0.26 mL) at −55 °C, and the mixture was stirred for 1 h. A solution of **8** (86 mg, 0.31 mmol) in THF (1 mL) was added to the mixture. After 1.5 h, the reaction was quenched with aqueous NH_4Cl, and the mixture was extracted with Et_2O (3 × 10 mL). The combined organic layers were washed with water (3 ×) and brine, dried, and concentrated. The residue thus formed was purified by silica-gel chromatography (15 g, ether/hexane = 1:10 then 1:1) to afford the coupling product (216 mg). This product (110 mg) was treated with CH_2Cl_2 (10 mL) and MS4Å (500 mg) at room temperature. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (10 mg) was added to this mixture every 5 min until the starting material was consumed. The mixture was poured into aqueous saturated $NaHCO_3$, and the aqueous layer was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were dried and concentrated to give a residue, which was purified by silica-gel chromatography (5 g, ether/hexane = 1:5) to afford **24** (82 mg, 75%, 2 steps). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 31.5 (d, J = 39.4 Hz, C21), 45.0 (d, J = 39.3 Hz, C19), 71.3 (d, J = 39.4 Hz, C22), 74.4 ppm (d, J = 39.3 Hz, C18); MS (ESI Q-TOF): m/z calcd for $C_{32}{}^{13}C_4H_{67}O_6S_2Si_2$: 719.4052 $[M+H]^+$; found: 719.4006.

6: TBAF (1 M solution in THF, 0.46 mL, 0.46 mmol) was added to a solution of **24** (82 mg, 0.11 mmol) in MeCN (3 mL). The mixture was stirred at 60 °C for 4 h, and the solvent was removed. The resulting residue was purified by silica-gel chromatography (5 g, ethyl acetate/hexane = 1:1 then ethyl acetate) to give diol **25** (51 mg, 91%). Diol **25** (48 mg, 0.10 mmol) was dissolved in THF (2 mL), and this solution was cooled to 0 °C. *n*BuLi (1.69 M solution in hexane, 0.14 mL, 0.22 mmol) was added to the mixture, which was stirred for 15 min. A solution of *p*-toluenesulfonyl chloride (29 mg, 0.15 mmol) in THF (1.5 mL) was added. After the mixture was stirred for 20 min, the reaction was quenched with phosphate buffer (pH 7) and the mixture extracted with Et_2O (3 × 15 mL). The combined organic layer was washed with aqueous saturated $NaHCO_3$, water (3 ×), and brine, dried, and then concentrated. The resulting residue was purified by silica-gel chromatography (4 g, ether/hexane = 1:5 then 1:2) to give **6** (34 mg, 74%). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ = 30.5 (d, J = 45.2 Hz, C21), 41.7 (d, J = 39.2 Hz, C19), 52.8 (d, J = 45.2 Hz, C22), 71.5 ppm (d, J = 39.2 Hz, C18); MS (ESI Q-TOF): m/z calcd for $C_{20}{}^{13}C_4H_{37}O_5S_2$: 473.2217 $[M+H]^+$; found: 473.2200.

2a: ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 0.80 (d, J = 6.5 Hz, 3H, 7-CH_3), 0.89 (d, J = 7.0 Hz, 3H, 13-CH_3), 0.97 (d, J = 7.0 Hz, 3H, 25-CH_3), 0.98 (d, J = 7.0 Hz, 3H, 25-CH_3), 1.00 (d, J = 6.5 Hz, 3H, 15-CH_3), 1.10 (d, J = 7.0 Hz, 3H, 3-CH_3), 1.11 (m, 3H, 19-CH_3), 1.20–1.70 (m, 17H, 4a-H, 4b-H, 5a-H, 5b-H, 7-H, 8a-H, 8b-H, 9a-H, 9b-H, 11a-H, 11b-H, 12a-H, 15-H, 16a-H, 16b-H, 17a-H, 17b-H), 1.83 (m, 1H, 13-H), 2.01 (m, 1H, 12b-H), 2.11 (m, 1H, 25-H), 2.15 (s, 3H, 1-H), 2.27 (d, J = 1.0 Hz, 3H, 5′-CH_3), 2.53 (sext, J = 7.0 Hz, 1H, 3-H), 2.67 (dm, $^1J_{H,C}$ = 144.0 Hz, 1H, 19-H), 2.67 (dm, $^1J_{H,C}$ = 128.0 Hz, 1H, 21a-H), 2.77 (dd, J = 16.5, 10.0 Hz, 1H, 2′a-H), 2.92 (dd, J = 16.5, 3.0 Hz, 1H, 2′b-H), 2.99 (ddd, J = 14.5, 8.0 Hz, $^1J_{H,C}$ = 128.0 Hz, $^2J_{H,C}$ = - 6.5 Hz, 1H, 21b-H), 3.16 (td, J = 10.0, 2.5 Hz, 1H, 6-H), 3.28 (m, 1H, 23-H), 3.28 (dd, J = 10.0, 2.0 Hz, 1H, 14-H), 3.44 (s, 3H, OCH_3), 3.70 (dm, $^1J_{H,C}$ = 144.0 Hz, 1H, 18-H), 4.35 (dm, $^1J_{H,C}$ = 144.0 Hz, 1H, 22-H), 5.10 (td, J = 6.0, $^3J_{H,C}$ = 4.5 Hz, 1H, 24-H), 5.21 ppm (m, 1H, 3′-H); ^{13}C NMR (150.9 MHz, $CDCl_3$): δ = 10.1, 11.0, 13.7 (d, J = 33.0, Hz, 19-CH_3), 16.2, 16.7, 17.9, 18.0, 19.4, 26.8, 27.4, 27.6, 28.1, 28.1, 28.7, 29.1. 30.2, 30.7, 31.4 (d, J = 38.6, Hz, C17), 34.8, 34.8, 36.0, 41.0, 45.8 (dd, J = 39.9, 10.3 Hz, C21), 47.3, 52.4 (dd, J = 37.3, 10.3 Hz, C19), 59.1, 63.9, 66.4 (d, J = 39.9 Hz, C22), 74.3 (d, J = 37.3 Hz, C18), 74.3, 74.8, 76.5, 80.6 (d, J = 43.0 Hz, C23), 95.7, 142.1, 143.0, 164.8, 165.8, 169.5, 213.1, 215.3 ppm (t, J = 40.0 Hz, C20); MS (FAB) (*m*-nitrobenzyl alcohol, NaI): m/z = 793 $[M+Na]^+$; HRMS (FAB) (glycerol): m/z calcd for $C_{37}{}^{13}C_4H_{67}O_{13}$: 771.4716 $[M+H]^+$; found: 771.4710.

2b: ^{13}C NMR (150.9 MHz, D_2O): δ = 47.3 (dd, J = 40.3, 10.0 Hz, C21), 53.4 (dd, J = 37.3, 10.0 Hz, C19), 66.3 (d, J = 40.3 Hz, C22), 74.1 ppm (d, J = 37.2 Hz, C18).

Preparation and purification of PP1γ: A full-length cDNA-encoding PP1γ sequence was expressed in *E. coli* (kindly provided by Dr. Patricia Cohen, University of Dundee, Dundee, UK) as described by Alessi et al.[28] All purification procedures were performed at 4 °C. After the plasmid was formed, the cells were harvested by centrifugation for 15 min at 4000*g*. The pellet was lysed in HEPES/NaOH buffer (pH 7.5, 50 mM, 200 mL) containing KCl (100 mM), glycerol (5% *v*/*v*), ethylenediamine tetraacetic acid (EDTA; 1 mM), dithiothreitol (DTT; 2 mM), phenylmethylsulphonyl fluoride (0.1 mM), $MnCl_2$ (2 mM), and benzamidine (1 mM). After the suspension was sonicated, it was immediately centrifuged for 30 min at 40000*g*. The supernatant was filtered through 5.0-μm, 1.0-μm, and 0.45-μm cellulose nitrate membrane filters and loaded onto a HiPrep 16/10 Q-XL column (1.6 cm × 10 cm, Pharmacia LKB Biotechnology) (2 ×) equilibrated triethanolamine/HCl buffer (pH 7.5, 25 mM) containing $MnCl_2$ (1 mM), ethylene glycol-bis(β-aminoethyl ether)tetraacetic acid (EGTA; 0.1 mM), glycerol (5% *v*/*v*), β-mercaptoethanol (βME; 0.10%), and brij-35 (0.03%) with NaCl (100 mM). The column was developed with a linear gradient for 80 min from 100 to 800 mM NaCl in triethanolamine/HCl buffer at a flow rate of 5 mL min^{-1}. The fractions containing PP were eluted in the range 250–350 mM NaCl. The collected fraction was concentrated to 20 mL by ultrafiltration (Amicon YM 10, 10 kDa pore) and exchanged against triethanolamine/HCl buffer with NaCl (100 mM). The solution was loaded onto a heparin sepharose column (1.6 cm × 10 cm, Pharmacia LKB Biotechnology) (four columns in series) and equilibrated in triethanolamine/HCl buffer with NaCl (100 mM). The column was developed with a linear gradient for 80 min from 100 to 800 mM NaCl in triethanolamine/HCl buffer at a flow rate of 4 mL min^{-1}. The fractions containing PP were eluted in the range 400–450 mM NaCl. Ground ammonium sulfate (25% *w*/*v*) was slowly added to the fraction, and the mixture was applied to a phenyl sepharose H/C column (2.6 cm × 10 cm, Pharmacia LKB Biotechnology) and equilibrated in triethanolamine/HCl buffer with ammonium sulfate (25%). The column was developed with a linear gradient for 200 min from 25 to 0% ammonium sulfate in triethanolamine buffer followed by isocratic elution (0%) for 200 min at the rate of 5 mL min^{-1}. The fractions containing PP were combined and concentrated to 4 mL by ultrafiltration. The solution was loaded onto a sephacryl S200HR 26/60 column (2.6 cm × 60 cm, Pharmacia LKB Biotechnology). The column was eluted with triethanolamine buffer at a flow rate of 0.6 mL min^{-1}. The portions of eluate (15 mL) with activity were combined and applied directly to a Mono Q column (1.6 cm × 10 cm, Pharmacia LKB Biotechnology) and equilibrated in triethanolamine buffer. The column was developed with a linear salt gradient for 30 min from 0 to 800 mM NaCl in triethanolamine/HCl buffer at the flow rate of 4 mL min^{-1}. The portions of eluate (8 mL) were combined, dialyzed overnight against triethanolamine buffer containing glycerol (50% *v*/*v*), and stored at −20 °C. The molar concentration of PP1γ was determined by the titration procedure described by Takai and Mieskes.[30] The purified sample of PP1γ gave a single spot at pH 6.0 and an apparent molecular mass of 37 kDa when subjected to 2D electrophoresis.

Preparation of the PP1γ/inhibitor complex: The solution of PP1γ (4.88 mg, 250 μL) in the aforementioned buffer was centrifuged at 4 °C for 1 h at 3000*g* with a Centricon YM-10 centrifuge followed by dilution with $[D_{11}]$Tris/DCl/D_2O buffer (30 mM $[D_{11}]$Tris, 2 mM $[D_{10}]$DTT, 200 mM NaCl, pH 7.5). The solution of PP1γ was exchanged against $[D_{11}]$Tris/DCl/D_2O buffer by repetition of this procedure. A solution of **2b** (0.2 mg, 0.26 μmol) and **4′** (0.1 mg, 0.18 μmol) as internal marker in $[D_6]$DMSO/D_2O (10%, 0.2 mL) was added to this solution of PP1γ (4.88 mg) in $[D_{11}]$Tris/DCl/D_2O buffer (0.7 mL), and the mixture was incubated at 25 °C for 15 min. DMSO used to dissolve the inhibitor. An aliquot of this solution (0.6 mL) was used for NMR spectroscopy of the PP1γ/inhibitor (0.5:1) complex. A solution of PP1γ (0.1 mL, 7.0 mg mL^{-1}) was added to the solution of the PP1γ/inhibitor (0.5:1) complex (0.15 mL), and the mixture was adjusted to 0.6 mL with $[D_{11}]$Tris/DCl/D_2O buffer. After incubation at 25 °C for 15 min, this solution was used for NMR spectroscopy of the PP1γ/inhibitor (0.75:1) complex. A solution of natural **1b** (5 μL, 6.6 mg mL^{-1}) in $[D_{11}]$Tris/DCl/D_2O buffer was added to the solution of the PP1γ/inhibitor (0.75:1) complex (0.6 mL). After incubation at 25 °C for 15 min, this solution was used for NMR spectrosco-

py of the PP1γ/^{13}C-labeled inhibitor/unlabeled inhibitor (0.75:1:1) complex (0.6 mL).

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