X-ray analysis on the nanogram to microgram scale using porous complexes

Yasuhide Inokuma¹, Shota Yoshioka¹, Junko Ariyoshi¹, Tatsuhiko Arai¹, Yuki Hitora², Kentaro Takada², Shigeki Matsunaga², Kari Rissanen³ & Makoto Fujita¹

X-ray single-crystal diffraction (SCD) analysis has the intrinsic limitation that the target molecules must be obtained as single crystals. Here we report a protocol for SCD analysis that does not require the crystallization of the sample. In our method, tiny crystals of porous complexes are soaked in a solution of the target, such that the complexes can absorb the target molecules. Crystallographic analysis clearly determines the absorbed guest structures along with the host frameworks. Because the SCD analysis is carried out on only one tiny crystal of the complex, the required sample mass is of the nanogram-microgram order. We demonstrate that as little as about 80 nanograms of a sample is enough for the SCD analysis. In combination with high-performance liquid chromatography, our protocol allows the direct characterization of multiple fractions, establishing a prototypical means of liquid chromatography SCD analysis. Furthermore, we unambiguously determined the structure of a scarce marine natural product using only 5 micrograms of the compound.

Chemists need reliable methods to analyse and determine molecular structures. Nuclear magnetic resonance (NMR) and mass spectrometry are indispensable tools in daily chemical research for rapidly analysing molecular structures, but, strictly speaking, they provide only speculative molecular structures that are sometimes assigned incorrectly. However, X-ray SCD provides direct structural information at the atomic level and is recognized as the most reliable structure determination method¹⁻³. Unfortunately, X-ray SCD has some critical limitations. First, the crystallization of samples before measurement can not be automated and usually requires a time-consuming trial-and-error procedure. Second, the method is in principle not applicable to non-crystalline molecules. In this Article, we describe an advance in crystallographic analysis based on a new X-ray analysis protocol that does not require the crystallization of the sample molecules themselves.

Our idea is to use networked porous metal complexes⁴⁻⁷ as 'crystalline sponges'8. Owing to the high molecular-recognition ability of the pores, the crystalline sponges can absorb target sample molecules from their solution into the pores, rendering the incoming molecules regularly ordered in the crystal. Accordingly, the molecular structure of the absorbed guest will be displayed, along with the host framework, by the crystallographic analysis of the networked porous complexes. We emphasize that even trace amounts of samples ($<0.1 \,\mu g$) can be analysed by this method because the experiment can be performed with only one tiny crystal (<0.1 mm to a side). In the following discussion, we thus describe the crystallographic analysis of non-crystalline compounds and nanogram-microgram-scale X-ray crystallography based on our method. The great advantage of trace-amount X-ray analysis is particularly emphasized by its application to liquid chromatography SCD analysis (see below), where high-performance liquid chromatography (HPLC) fractions are directly collected by the crystalline sponge and analysed by X-ray crystallography. Furthermore, we successfully determine the structure of a scarce marine natural product, miyakosyne A, including the absolute configuration of its chiral centre, which could not be determined by conventional chemical and spectroscopic methods.

X-ray crystallography of liquid samples

In this study, two networked complexes are used as crystalline sponges: $\{[(Co(NCS)_2)_3(1)_4] \bullet x(solvent)\}_n$ (2; ref. 8) and $\{[(ZnI_2)_3(1)_2] \bullet x(solvent)\}_n$ (3; ref. 9). Both of these can be easily prepared from tris(4-pyridyl)-1,3,5-triazine (1) and a metal salt $(Co(NCS)_2 \text{ and } ZnI_2, \text{ respectively})$. Complex 2 has a three-dimensionally networked cage framework, the unit structure of which is identical to that of a discrete M₆L₄ cage host¹⁰ (4; M = ethylenediamine-capped Pd(II) ion, L = ligand 1). The framework of 3 is completely different from that of 2, but the void spaces of both 2 and 3 show strong binding properties for incoming guest molecules, similar to the molecular recognition by discrete cage 4 in solution. The as-synthesized complexes 2 and 3 contain solvents in the void, which can be exchanged for other guests by soaking the crystals of 2 and 3 in a guest solution. The crystallographic analysis of guestexchanged 2 and 3 has been reported^{8,11-14}, but primarily a large excess of solid guests have been examined. Focusing on the crystallographic analysis of non-crystalline guests on a small scale, we first developed a procedure for one-crystal-scale guest uptake with only a drop of liquid guest (Fig. 1a). When a crystal of 2 was brought into contact with a drop of cyclohexanone (5) at room temperature (25 °C), rapid guest penetration occurred. After 2 d, the crystal was subjected to an X-ray diffraction experiment. As expected, the crystal data clearly showed the molecular structure of 5 packed within the cavity of the unit cage in a host/guest ratio of 1:4 (Fig. 1b). In a similar manner, complex 3 also enclathrated a liquid guest, isoprene (6), which is a highly volatile alkene with a melting point below -145 °C (Fig. 1c). Once absorbed into a crystal of 3, guest 6 was no longer volatile and its structure was easily determined by X-ray crystallography without any special treatment.

It is worth mentioning that the absorbed guests are rarely observed by X-ray analysis with common porous coordination networks^{4–7,15–18}. Such networks are mostly characterized by their permanent vacuum pores, which rapidly absorb small molecules (normally gases), presumably in a kinetic way. In contrast, the void spaces of as-synthesized crystals of **2** and **3** are filled with highly fluid solvent molecules. Guest

¹Department of Applied Chemistry, Graduate School of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan. ²Laboratory of Aquatic Natural Products Chemistry, Graduate School of Agricultural and Life Science, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan. ³Department of Chemistry, NanoScience Center, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland.



Figure 1 X-ray crystallographic observation of liquid guest molecules using crystalline sponges. a, Schematic outline for the preparation of a guestincluded network complex: a single piece of crystal **2** or **3** was treated for 2 d with a drop of a liquid guest and subjected to X-ray data collection. The experiment with isoprene was carried out in a sealed vial to avoid the

evaporation of the guest. **b**, X-ray crystal structure of cyclohexanone (5) observed in the cavities of **2**. **c**, Structure of isoprene (6) observed in the pores of **3**. In the network structures, liquid guests are shown as space-filling models. Thermal ellipsoids are drawn at the 50% probability level.

molecules can slowly penetrate these 'wet' cavities by guest exchange, and are concentrated at the molecular-recognition pockets surrounded by ligand **1**. A characteristic of the strong host–guest interaction in the crystals of **2** and **3** lies in panel ligand **1**, which attracts various guests onto its electron-deficient π -plane^{19,20}. The entire process takes place under thermodynamic control, similar to host–guest complexation in



Figure 2 | Nanogram-scale guest inclusion with a crystal of crystalline sponge 3. a, Photograph of a microvial including a single crystal of crystalline sponge 3 (solvent, cyclohexane) and a CH_2Cl_2 /cyclohexane (1:9) solution of guaiazulene 7 (500 ng/50 µl). The schematic illustration represents the bottom

of the vial highlighted with a red box in the left picture. **b**, Microgram of the single crystal of **3** placed in a microvial. **c**, Network structure of guaiazulene (7) included in crystalline sponge **3** and ORTEP drawing (at the 50% probability) of the guest **7**. (C, grey; H, white).



Figure 3 | Crystal structures of a variety of guests determined using a onecrystal-scale inclusion protocol. Guest inclusion and X-ray diffraction measurement were conducted for each compound with 5 μ g of sample and one

solution. As a result, the geometry of the included guests is well equilibrated and regularly ordered, making the crystallographic analysis of the accommodated guests possible. The equilibration of guest geometry, which is an easily overlooked point in rendering the guests in order, does not normally occur in kinetic guest capture by the permanent pores of coordination networks, so the guests are severely disordered and have rarely been observed by X-ray analysis²¹⁻²⁵.

Nanogram-microgram-scale X-ray analysis

A great advantage of this method is particularly clear when it is applied to the structural analysis of trace amounts of samples. Given that the experiment is performed with only one tiny crystal, the required sample amount is estimated to be of the nanogrammicrogram order. Because the guest-accessible void of **3** accounts for ~50% of the lattice, one tiny crystal (typically 100 µm to a side; ~1 µg) theoretically requires only ~0.5 µg of organic guest (density, ~1 g cm⁻³), indicating that X-ray crystallographic analysis can be performed on nanogrammicrogram scales. Furthermore, if only one of the recognition sites in **3** firmly traps the guest, it is not necessary for the rest of the void space to bind the guest. Thus, the average occupancy of the guest in the crystal can be much lower than ~100%, further decreasing the lower limit of the sample amount.

Encouraged by the theoretical estimation, we examined the one-crystal-scale guest inclusion using only 500 ng of guaiazulene (7), a natural hydrocarbon. A cube-shaped crystal of **3** (solvent = cyclohexane; $60 \times 60 \times 70 \,\mu\text{m}^3$) was placed in a microvial with 45 μ l of cyclohexane. A CH₂Cl₂ solution of guest 7 (500 ng, 5 μ l) was added to the supernatant and the microvial was capped with a screw cap with a pinhole as an exhaust outlet (Fig. 2). The solvent slowly evaporated through the pinhole in an incubator maintained at 45 °C over 2 d. As the volume of supernatant decreased, the crystal of **3**, which was initially colourless, gradually turned dark blue, indicating the concentration of 7 in the

network crystal **3**. **a**, Chemical structures of guests. Me, methyl. **b**, Inclusion crystals prepared with $5 \mu g$ of sample. **c**, ORTEP representation of the crystal structures of the guests in the pores of **3**.

crystal. The crystal was removed from the vial and directly mounted onto an X-ray diffractometer to collect the diffraction data. The crystal structure thus obtained showed 7 trapped in the pores with 60% occupancy (Fig. 2c). Importantly, two methyl and one isopropyl substituent on the azulene core were readily apparent from the observed electron density map.

From the occupancy of 7, the total amount of the guest actually included in the crystal was estimated to be only 26 ng, which accounts for less than 10% of the guest added to the supernatant. This result motivated us to reduce the guest amount further. Surprisingly, when only 80 ng of guest 7 was used with a crystal of $3 (80 \times 80 \times 80 \,\mu\text{m}^3)$, guest 7 was still clearly observed. Considering that the experiment was carried out using a laboratory X-ray machine, we presume that crystallography is possible with synchrotron X-ray experiments even on a mass of <10 ng.

For practical reasons, a typical protocol for trace-amount crystallography was established with 5 µg of sample (Methods Summary). Six appropriate samples were selected and one of us (S.Y.) performed the trace-amount crystallography with only \sim 5 µg of each sample and without any knowledge of the structures. In this blind test, three structures were fully determined from only the diffraction data (Fig. 3). The other three were initially flawed because of atom misassignment, symmetry problems and guest disorder, which are common problems in crystallographic analysis. However, the incorrect structures could be easily corrected using only the mass spectrometric data (molecular weight information). Thus, like common crystallographic analysis, the refined crystal structures should be supported by other methods of structural analysis such as mass and NMR spectrometry. The successful structural determination of these compounds indicates that the scope of guests can be considerably extended to include polycyclic, non-aromatic and non-planar molecules.



Figure 4 | The crystal structure of a chiral guest, santonin, trapped in a crystalline sponge. a, Network structure of the clathrate comprising santonin

(8) and complex 3. b, ORTEP drawing (50% probability) of the santonin trapped in the pore, and chemical structure of santonin.



Figure 5 | **LC–SCD analysis of natural flavonoids. a**, Sketch of LC–SCD analysis. Each fraction isolated by analytical HPLC is poured into a microvial that contains a single crystal of crystalline sponge. **b**, HPLC chromatogram of PMFs extracted from the peel of *Citrus unshiu*. **a.u**., arbitrary units. **c–e**, X-ray

Absolute structure determination

The determination of the absolute configuration of chiral compounds is one of the most difficult analyses of molecular structures^{26–28}. NMR and other spectroscopic methods can determine in principle only relative stereochemistry. X-ray crystallography is the only method that can determine the absolute configuration of chiral molecules, on the basis of the anomalous scattering effects of heavy atoms^{29,30}. Surprisingly, we were able to determine the absolute structure of a trace amount $(5 \mu g)$ of a chiral compound because the heavy atoms (Zn and I) in the host framework (3) showed clear anomalous scattering. As-synthesized coordination network 3 has an achiral space group (C2/c), but when treated with 5 µg of santonin (8), an anthelminthic drug bearing four chiral centres³¹, the space group changed to chiral $P2_1$ owing to the enclathration of the chiral guest in the pores. The refined absolute structure was validated by the Flack parameter (0.092(18); parenthetical error, s.d.) and the stereochemistry of 8 was unambiguously determined (Fig. 4). It is noteworthy that, in contrast to common absolute structure determinations³²⁻³⁴, the chemical introduction of heavy atoms on the guest skeleton is unnecessary because the host framework contains heavy atoms and shows enhanced anomalous scattering effects. We believe that our method is the most practical and reliable method for the determination of the absolute structure of chiral compounds.

LC-SCD analysis

The requisite guest amount in our protocol matches the separation scale of analytical HPLC (a few micrograms). This prompted us to propose a combination of liquid chromatography and SCD, namely LC–SCD analysis. In this analysis, a trace amount of a sample mixture is separated by HPLC and each fraction is directly treated with crystalline sponge **3**. Subsequently the molecular structure of each fraction is analysed by X-ray crystallography. As a proof of concept, we considered the structural determination of a series of polymethoxyflavones (PMFs) that are trace components in orange peel^{35,36}. A crude mixture of PMFs (~30 µg) extracted from a piece of air-dried orange

crystal structures of fraction A, nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) (**9**; **c**); fraction B (3,5,6,7,8,3',4'-heptamethoxyflavone) (**10**; **d**); and fraction C, tangeritin (5,6,7,8,4'-pentamethoxyflavone) (**11**; **e**) obtained by LC–SCD analysis. (C, black; O, red; H, grey).

peel, Citrus unshiu, was separated by analytical HPLC to give three main components, A, B and C (Fig. 5), that weighted 6, 7 and 5 µg, respectively. Each fraction was then directly subjected to the guest inclusion conditions with one single crystal of 3. The X-ray crystal structures of the three samples all showed a flavone ring trapped at a similar position in the host pores. On the basis of the X-ray structures, components A, B and C were identified as PMFs9, 10, and 11, respectively. These structures are in good accordance with their mass data (Supplementary Information). It is worth noting that, despite the PMFs' small, simple structures, the determination of their substitution positions is unexpectedly difficult even with the use of NMR spectroscopy. Considering the applicable range of our X-ray protocol, the LC-SCD analysis will be a powerful tool for the rapid characterization of multiple components with much higher structural reliability than liquid chromatography mass spectrometry and LC-NMR techniques.

Complete stereochemistry of miyakosyne A

Using our method, we took on the considerable challenge of determining the structure of miyakosyne A, a scarce natural product. Miyakosyne A (**12**), a marine natural product recently isolated from a marine sponge *Petrosia* sp., has three chiral centres on its main alkyl chain³⁷ (Fig. 6a). Although the absolute configurations at C3 and C26 have been previously determined to be 3*R* and 26*R*, respectively, conventional spectroscopic and chemical methods are no longer effective for the determination of the absolute configuration at C14 because the difference between the two long alkyl groups on C14 is only one methylene unit. X-ray crystallography is expected to be the only method that can determine the C14 configuration, but preparation of a single crystal of **12** from only a limited amount is an extremely challenging task. Hence, we applied our method to the full characterization of miyakosyne A to determine the absolute configuration at C14.

A tiny crystal of **3** (approximately $100 \times 100 \times 100 \,\mu\text{m}^3$) was treated with 5 µg of miyakosyne A (**12**) and subjected to a diffraction



Figure 6 | Structural determination of miyakosyne A. a, Chemical structure of miyakosyne A (12). Numbers indicate carbon atoms; asterisks indicate asymmetric carbon atoms. b, Orientation of miyakosyne A enclathrated in the pore of crystalline sponge 3. The host framework 3 is drawn as a brown surface,

and the guest is represented as a ball-and-stick model. (C, green; O, red; H, white) c, The absolute structure of miyakosyne A determined by relative configuration to C3 and C26. The chiral centre C14 is drawn in magenta.

study. The crystal structure was successfully solved in the chiral C2 space group and revealed all the stereochemistry of 12. On the basis of the known 3R and 26R configurations, the C14 configuration of 12 was determined to be S (Fig. 6b, c). Because the occupancy of the solved guest 12 was \sim 50% due to disordered guests and remaining solvents in the pores, we carefully checked the validation of the crystal structure. We found that the observed electron density map $(2F_{0}-F_{c})$ can be well superimposed on the refined structure of (3R,14S,26R)-12, and that when least-squares refinement was performed with an incorrectly modelled 14R form without any restraints around C14, the refinement did not converge to this configuration but converged finally to the 14S form. This result is a striking example of the structural determination of a scarce natural product and shows that our method can be expanded in scope to cover the structural elucidation of even large and conformationally flexible compounds. A variety of different types of compound can be quickly and readily analysed in a similar way.

Conclusion

Our method solves the real and intrinsic problems of X-ray crystallography and transforms it into a rapid and convenient method for the analysis of molecular structures using only a trace amount of sample. The following features are worthy of special attention. First, the crystallization step, which is the bottleneck of the X-ray analysis protocol, becomes unnecessary. Therefore, the crystallographic study of molecular structures is drastically accelerated and is now applicable to the analysis of liquid or even volatile compounds. Second, crystallographic analysis can be preformed on the nanogram-microgram scale. Thus, in terms of sensitivity, X-ray analysis overwhelmingly dominates NMR analysis and is even comparable to mass spectrometry. The combination of HPLC and single-crystal X-ray analysis (LC-SCD analysis) has been achieved. Third, the determination of the absolute configuration of chiral molecules can be easily carried out without any chemical modification of the sample molecules.

The slow diffusion of guests into the solvent-filled voids of the crystalline sponges is a particularly important process that renders the guests thermodynamically well equilibrated at the specific molecular-recognition sites of the hosts. Accordingly, the guests are regularly ordered and made observable by crystallographic analysis. This is not always the case with common porous coordination networks, in which the permanent pores absorb guests in presumably a

kinetic way. Because the crystalline sponges used here are easily prepared from commercially available components, the nanogrammicrogram-scale crystallographic analysis of non-crystalline molecules is already practicable. We expect that our method will be applicable to microanalysis in natural product chemistry, food and perfume science, drug discovery, forensic science, and, most importantly, the daily research of synthetic chemists³⁸⁻⁴⁰. We believe that many natural and synthetic compounds that chemists have almost given up hope of analysing crystallographically will be easily and precisely characterized by this method.

METHODS SUMMARY

A cube-shaped crystal of 3 (approximately $100 \times 100 \times 100 \,\mu\text{m}^3$) was placed in the bottom of a microvial with 45 µl of cyclohexane. A solution of guest compound (5 µg) in dichloromethane (5 µl) was added to the supernatant, and the microvial was capped with a screw cap pierced with a hollow needle (internal diameter, 0.8 mm). The solvent of the supernatant was slowly evaporated in an incubator maintained at 45 °C over 2 d at a rate of $\sim 1 \,\mu l \, h^{-1}$. The guest inclusion crystal was directly mounted onto a single-crystal X-ray diffractometer with a cryoloop and the diffraction data were collected. The crystal structures were solved and refined by using the SHELX 97 program. Further details and other methods are available in Supplementary Information.

Full Methods and any associated references are available in the online version of the paper.

Received 13 November 2012; accepted 5 February 2013.

- 1
- Ooi, L. *Principles of X-Ray Crystallography* (Oxford Univ. Press, 2010). Sheldrick, G. M. A short history of *SHELX. Acta Crystallogr. A* **64**, 112–122 (2008). 2. 3. Ohashi, Y. in Models, Mysteries and Magic of Molecules (eds Boeyens, J. C. A. &
- Ogilvie, J. F.) 109-113 (Springer, 2008). Batten, S. R. & Robson, R. Interpenetrating nets: ordered, periodic entanglement.
- Angew. Chem. Int. Ed. 37, 1460-1494 (1998).
- 5 Kitagawa, S., Kitaura, R. & Noro, S. Functional porous coordination polymers. Angew. Chem. Int. Ed. 43, 2334–2375 (2004).
- Yaghi, O. M. et al. Reticular synthesis and the design of new materials. Nature 423, 705-714 (2003).
- Fujita, M., Kwon, Y. J., Washizu, S. & Ogura, K. Preparation, clathration ability and 7. catalysis of a two-dimensional square network material composed of cadmium(II) and 4.4'-bipyridine. J. Am. Chem. Soc. 116, 1151-1152 (1994).
- Inokuma, Y., Arai, T. & Fujita, M. Networked molecular cages as crystalline sponges for fullerenes and other guests. Nature Chem. 2, 780-783 (2010).
- 9. Biradha, K. & Fujita, M. A springlike 3D-coordination network that shrinks or swells in a crystal-to-crystal manner upon guest removal or readsorption. Angew. Chem. Int. Ed. 41, 3392-3395 (2002).
- 10. Fujita, M. et al. Self-assembly of ten molecules into nanometre-sized organic host frameworks. Nature 378, 469-471 (1995).



- Inokuma, Y., Kojima, N., Arai, T. & Fujita, M. Bimolecular reaction via the successive introduction of two substrates into the crystals of networked molecular cages. J. Am. Chem. Soc. 133, 19691–19693 (2011).
- Ohmori, O., Kawano, M. & Fujita, M. Crystal-to-crystal guest exchange of large organic molecules within a 3D coordination network. J. Am. Chem. Soc. 126, 16292–16293 (2004).
- Haneda, T., Kawano, M., Kojima, T. & Fujita, M. Thermo-to-photo-switching of the chromic behavior of salicylideneanilines by inclusion in a porous coordination network. *Angew. Chem. Int. Ed.* 46, 6643–6645 (2007).
- Ohara, K., Kawano, M., Inokuma, Y. & Fujita, M. A porous coordination network catalyzes an olefin isomerization reaction in the pore. J. Am. Chem. Soc. 132, 30–31 (2010).
- Férey, G. Hybrid porous solids: past, present, future. Chem. Soc. Rev. 37, 191–214 (2008).
- Li, J.-R., Kuppler, R. J. & Zhou, H.-C. Selective gas adsorption and separation in metal–organic frameworks. *Chem. Soc. Rev.* 38, 1477–1504 (2009).
- Chen, B., Xiang, S. & Qian, G. Metal-organic frameworks with functional pores for recognition of small molecules. Acc. Chem. Res. 43, 1115–1124 (2010).
- Kondo, M. et al. Three-dimensional framework with channeling cavities for small molecules: {[M₂(4,4'-bpy)₃(NO₃)₄]•xH₂O}_n (M = Co, Ni, Zn). Angew. Chem. Int. Edn Engl. 36, 1725–1727 (1997).
- Yoshizawa, M., Klosterman, J. K. & Fujita, M. Functional molecular flasks: new properties and reactions within discrete, self-assembled hosts. *Angew. Chem. Int. Ed.* 48, 3418–3438 (2009).
- Inokuma, Y., Kawano, M. & Fujita, M. Crystalline molecular flasks. Nature Chem. 3, 349–358 (2011).
- 21. Li, Q. W. et al. Docking in metal-organic frameworks. Science 325, 855–859 (2009).
- Kim, H., Chun, H., Kim, G.-H., Lee, H.-S. & Kim, K. Vapor phase inclusion of ferrocene and its derivative in a microporous metal-organic porous material and its structural characterization by single crystal X-ray diffraction. *Chem. Commun.* 2759–2761 (2006).
- Halder, G. J. & Kepert, C. J. In situ single-crystal X-ray diffraction studies of desorption and sorption in a flexible nanoporous molecular framework material. J. Am. Chem. Soc. 127, 7891–7900 (2005).
- Kawano, M. & Fujita, M. Direct observation of crystalline-state guest exchange in coordination networks. *Coord. Chem. Rev.* 251, 2592–2605 (2007).
- 25. Kitaura, R. et al. Formation of a one-dimensional array of oxygen in a microporous metal-organic solid. *Science* **298**, 2358–2361 (2002).
- 26. Cahn, R. Š., Ingold, C. & Prelog, V. Specification of molecular chirality. Angew. Chem. Int. Edn Engl. 5, 385–415 (1966).
- Seco, J. M., Quiñoá, E. & Riguera, R. The assignment of absolute configuration by NMR. Chem. Rev. 104, 17–118 (2004).
- Freedman, T. B., Cao, X., Dukor, R. K. & Nafie, L. A. Absolute configuration determination of chiral molecules in the solution state using vibrational circular dichroism. Chirality 15, 743–758 (2003).
- Bijvoet, J. M., Peerdeman, A. F. & van Bommel, A. J. Determination of the absolute configuration of optically active compounds by means of X-rays. *Nature* 168, 271–272 (1951).
- Flack, H. D. & Bernardinelli, G. Absolute structure and absolute configuration. Acta Crystallogr. A 55, 908–915 (1999).

- Corey, E. J. The stereochemistry of santonin, β-santonin, and artemisin. J. Am. Chem. Soc. 77, 1044–1045 (1955).
- Deschamps, J. R. X-ray crystallography of chemical compounds. *Life Sci.* 86, 585–589 (2010).
- Takayanagi, H., Sudou, M. & Ogura, H. Crystal structure of 1α,2β-dibromo-1,2dihydro-α-santonin. Anal. Sci. 7, 183–184 (1991).
- Inayama, S. *et al.* Unusual bromination of tetrahydro-(–)-α-santonins and new santonin isomers: X-ray crystal and molecular structure of 2β,14-dibromo-4α,5β,6β,11βH-tetrahydrosantonin. *J. Chem. Soc. Chem. Commun.* 495–496 (1980).
- Green, C. O., Wheatley, A. O., Osagie, A. U., Morrison, E. Y. S. A. & Asemota, H. N. Determination of polymethoxylated flavones in peels of selected Jamaican and Mexican citrus (*Citrus* spp.) cultivars by high-performance liquid chromatography. *Biomed. Chromatogr.* 21, 48–54 (2007).
- Han, S. et al. Isolation and identification of polymethoxyflavones from the hybrid Citrus, Hallabong. J. Agric. Food Chem. 58, 9488–9491 (2010).
- Hitora, Y., Takada, K., Okada, S. & Matsunaga, S. Miyakosynes A–F, cytotoxic methyl branched acetylenes from a marine sponge *Petrosia* sp. *Tetrahedron* 67, 4530–4534 (2011).
- Sampietro, D. A., Catalan, C. A. N. & Vattuone, M. A. Isolation, Identification, and Characterization of Allelochemicals/Natural Products (Science Publ., 2009).
- Croue, J.-P., Korshin, G. V. & Benjamin, M. M. Characterization of Natural Organic Matter in Drinking Water 73–374 (Am. Water Works Assoc., 1999).
- Ahuja, S. & Alsante, K. Handbook of Isolation and Characterization of Impurities in Pharmaceuticals (Academic, 2003).

Supplementary Information is available in the online version of the paper.

Acknowledgements This research was supported by Grants-in-Aid for Specially Promoted Research (24000009) and Young Scientists (B) (23750146), and by the CREST project of the Japan Science and Technology Agency. The experiment involving X-ray crystallography with 80 ng of guest molecules was performed using VariMax optics with a RAPID image plate detector system, courtesy of Rigaku Corporation. We thank M. Yamasaki and H. Sato for support for X-ray measurements.

Author Contributions Y.I. and M.F. designed the project, analysed results and wrote the manuscript. S.Y., J.A. and T.A. performed the experimental work and crystallographic analysis. Y.H., S.M. and K.T. selected and provided a natural product sample for analysis. K.R. confirmed the validity of the X-ray crystallographic analysis of all data.

Author Information The X-ray crystallographic coordinates for structures reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under deposition numbers CCDC 910380, 910381, 910382, 910383, 910384, 910385, 910386, 910386, 910387, 910388, 910389, 910390, 910391, 910392, 910393 and 910394. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre (http://www.ccdc.cam.ac.uk/data_request/cif). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.F. (mfujita@appchem.tu-tokyo.ac.jp).

METHODS

Chemicals. Solvents and reagents were purchased from TCI, WAKO Pure Chemical Industries and Sigma-Aldrich, and were used without any further purification except where noted.

Glassware. Microvials for this research were purchased from Waters (Deactivated Clear Glass 12×32 mm Screw Neck Max Recovery Vial; 1.5 ml). A syringe needle, Terumo NN-2116R (internal diameter, 0.80 mm), was used as an exhaust outlet for the cap of the microvial. An incubator (Fine FF-12) was used to maintain the temperature during guest inclusion.

Equipment. HPLC separation of microgram-scale natural products was performed on a Jasco UV-970 spectrometer equipped with a Jasco PU-980 pump and an ODS column Develosil ODS-5, (Nomura Chemical Co.). Elemental analysis was performed on a CHN/O/S elemental analyser (CE-440, Exeter Analytical). Mass spectra were recorded on a Bruker maXis spectrometer. Single-crystal X-ray diffraction data were collected on a Bruker APEX-II CCD diffractometer equipped with a focusing mirror (MoK_a radiation wavelength, 0.71073 Å) and an N₂ generator (Japan Thermal Engineering Co.) or a Rigaku VariMax with RAPID (CuK_a radiation wavelength, 1.54187 Å). Microscopic infra-red spectra were recorded on a Varian DIGILAB Scimitar instrument. For X-ray diffraction and microscopic infrared measurements, fluorolube was used as a protectant for the single crystals.

X-ray structural analysis. The structures were solved by direct methods and refined by full-matrix least-squares calculations on F^2 (the squared structure

factor), using the SHELX 97 program. Residual electron densities in the solvent-accessible void due to disordered solvent molecules were treated with the PLATON/SQUEEZE program. The quite large $(3e-9e \text{ Å}^{-3})$ residual electron density peaks causing checkCIF B level alerts in SQUEEZE are all located near the iodine atoms in the corresponding structures and could not be modelled with reasonably disordered atoms.

Plant material. *Citrus unshiu* was cultivated in Nishi-Uwajima, Ehime prefecture, Japan in 2011. The peel of *C. unshiu* was air-dried at room temperature over 1 week and ground into a fine powder with a lab mixer.

Inclusion of cyclohexanone (5) into a crystal of 2. On a glass plate, a crystal of **2** ($280 \times 280 \times 200 \ \mu m^3$) was soaked in a drop of cyclohexanone (**5**) ($5 \ \mu$ l) at room temperature. After standing for 2 d, the crystal was picked using a protectant and mounted onto the X-ray diffractometer. After collection of the diffraction data, the same crystal was subsequently analysed by microscopic Fourier transform infrared spectroscopy.

Inclusion of isoprene (6) into a crystal of 3. In a microvial, a crystal of 3 ($80 \times 50 \times 50 \ \mu\text{m}^3$) was soaked in a drop of isoprene (6) (5 μ l). Then the vial was sealed with a screw cap and allowed to stand for 2 d at room temperature. The crystal was then analysed as described above.

Nanogram-scale inclusion of guaiazulene (7). To a microvial containing a single crystal of **3** (approximately $80 \times 80 \times 80 \ \mu\text{m}^3$) and cyclohexane (45 μ l), we added a dichloromethane solution of guaiazulene (7) (80 ng/5 μ l or 500 ng/5 μ l). The crystal was heated at 45 °C for 2 d, as described in the general procedure.